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A study of the relationship between viability, culturability and virulence in environmental populations of Legionella pneumophila

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A STUDY OF THE RELATIONSHIP BETWEEN VIABILITY, CULTURABILITY AND VIRULENCE IN ENVIRONMENTAL POPULATIONS OF *LEGIONELLA PNEUMOPHILA*

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To Mom, Dad, Bill, Teriann and Craig

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INTRODUCTION

Legionella in Natural Environment

Members of the genus *Legionella* are thin, somewhat pleomorphic Gram-negative bacilli that measure 2 to 20 microns by 0.3 to 0.9 microns. They are aerobic, do not form endospores and are not encapsulated. Motility is by one or more straight or curved polar or lateral flagella; nonmotile strains are occasionally seen. Carbohydrates are neither fermented nor oxidized (Holt, 1994). Carbon and energy requirements can be met with nine amino acids (Tresh and Miller, 1981); there seems to be no vitamin requirements (Ristroph et al., 1981). Legionellae are ubiquitous in natural aquatic environments. Water samples have been collected from all parts of the country and examined for the presence of this organism. *Legionella pneumophila* and other legionellae have been isolated from rivers, streams, thermally polluted waters, natural thermal ponds and the shores of lakes. Wherever one finds water, one will find legionellae (Fliermans et al., 1979, 1981; Fliermans, 1985).

Legionella in Domestic Water Systems

Legionellae may survive water treatment and disinfection and pass intact through a distribution system. Kuchta et al. (1983, 1985) showed that these organisms are more resistant to chlorine than are coliform bacteria, and once legionellae are exposed to that disinfectant their resistance is increased. These organisms can become incorporated into amoebic cysts which require for eradication such a high chlorine concentration and long exposure time that the

disinfectant may be ineffective (Fliermans, 1996). In addition, these microbes enter a water system through broken or corroded piping, during repair of existing mains or installation of new mains. In older distribution systems deterioration of piping is often so severe that treated water comes in contact with soil, enabling organisms to enter from surface water. New water distribution systems or components that were not appropriately cleaned and disinfected before use may introduce legionellae into the system; serious outbreaks have occurred in newly opened buildings (Ware, 1989).

Within municipal drinking water systems the potential for *Legionella* growth may increase. The chemical environment affects the ability of the water to support multiplication, with low chlorine residuals, turbidity, organic carbon and certain other metals being important. Growth potential is most pronounced in the bottoms and stagnant corners of finished water reservoirs. Despite treatment with copper sulfate, these reservoirs typically support low-density algal populations during the warmer months (States et al., 1987).

Green algae, cyanobacteria, protozoa and heterotrophic bacteria have been shown to promote growth of *L. pneumophila.* When grown in association with various green algae and cyanobacteria in minimal-salts media with no organic compounds added, *L. pneumophila* increased in number (Pope et al., 1982). *L. pneumophila* was shown to adhere to the slime coat of *Fischerella,* and when the cyanobacterium was removed from coincubation mixtures, *L. pneumophila* colony forming units decreased (Bohach and Snyder, 1983). Amoebae support the

growth of *L. pneumophila* (Wadowsky et al., 1988); virulent strains of the bacterium can infect amoebae and grow intracellularly (Wadowsky et al., 1993). Five genera of amoebae and one genus of ciliated protozoa (*Tetrahymena*) have been shown to suppport the intracellular growth of *L. pneumophila* (Fields, 1993). Barker et al., (1993) showed that *L. pneumophila* grown within *Acanthamoeba* contained abundant quantities of a protein and fatty acid found in the host amoeba; these organics were not found in *L. pneumophila* grown in vitro. Wadowsky and Yee (1983, 1985) reported that nonLegionella bacteria present in plumbing systems may supply *L. pneumophila* with at least some of the amino acids required for its metabolism or enhance cryptic growth.

Legionnaires' Disease

Once inside a water distribution system, *Legionella* may cause Legionnaires' Disease, a severe form of pneumonia. Within the *Legionella* genus there are thirty-four species of which *Legionella pneumophilia* most commonly causes illness. *L. pneumophila* includes fourteen serogroups (States et al, 1990). Each serogroup is subdivided into serotypes. Ninety percent of all cases of Legionnaires' Disease are caused by serogroups 1, 3, 4 and 6 (Fliermans, 1996).

Legionnaires' Disease was first identified in 1976 after an outbreak at an American Legion convention in Philadelphia. Of the 4,400 attendees and some other individuals not directly associated with the convention, two hundred twenty-one became ill and thirty-four of these died (Fraser et al, 1977). The cause of the epidemic was unknown until later that year when the Center for Disease Control

isolated the responsible bacterium. Reexamination of bacterial specimens from earlier explosive outbreaks of pneumonia indicated *L. pneumophila* was not a new pathogen but had occurred uncharacterized for several years (McDade et al, 1979).

The symptoms of Legionnaires' Disease are a fever of 102-105°F, chills and cough. Occasionally there is abdominal pain, diarrhea and confusion. It can be difficult to distinguish from other forms of pneumonia. The symptoms progress rapidly. The recommended treatment is with the antibiotic erythromycin.

The disease can be contracted by breathing an aerosol contaminated with the bacteria. The potential host must inhale particles that are less than five microns in size so that the organisms reach the deepest parts of the lungs. The mist may come from rooftop cooling towers used in large air conditioning systems or showers, water faucets, whirlpools, fountains and vegetable sprayers in supermarkets (Fliermans, 1996). Dental-unit water lines are implicated as a source of infection (Atlas et al., 1995).

Accurate information on the incidence of Legionnaires' Disease is extremely limited. Estimates of the incidence of the disease have ranged from 10,000 to 100,000 cases annually in the United States. Cases reported to the Centers for Disease Control may be only a minute fraction of the total that occur. Consequently, estimates of incidence are derived from epidemiologic studies (Horwitz, 1993). The risk of contracting the disease depends on factors such as: the susceptibility of the people exposed, the number of organisms inhaled and the virulence of the bacteria. Some serotypes of *L. pneumophila* are virulent while

others are not (Young, 1996). Those at highest risk include the elderly, especially heavy smokers, transplant patients, those with a weakened immune system or individuals with chronic lung disease. According to CDC 1994 statistics the mortality rate for people who were infected outside of a hospital is about 20%; inside hospitals about 40% (Heinlein, 1996).

Although Legionnaires' Disease may develop in some healthy persons, these individuals usually contract a milder nonpneumonia form of the disease called Pontiac fever named after the city in Michigan where the first outbreak was recognized in 1968. This infection resembles acute influenza, including fever, headache, and severe muscle aches. Complete recovery usually occurs in two to five days without medical intervention (Glick et al.,1978; Ware, 1989; Winn, 1991). It is not clearly understood why the same organism causes two different clinical respones, Legionnaires' Disease and Pontiac fever.

The source of *Legionella* outbreaks is often elusive. In investigations of Legionnaires' disease outbreaks the source of infection is often difficult to identify. For example, in the October 1996 outbreak in Farmington, Michigan thirty people contracted the disease, four of which died. Public health workers looked for the source of the outbreak for three weeks. Because there appeared to be no single business or event all of the patients had in common, health officials theorized the contaminated mist came from an outdoor source. The source of the outbreak was attributed to the rooftop cooling tower of Cattleman's meat market in the area. The *Legionella* found in this cooling tower matched the type of

Legionella found in victims (Wendland, 1996; Wendland and Doran, 1996).

In 1983 fifteen persons developed Legionnaires' Disease in a hospital in Rhode Island. Initially it was thought the organism was present in the hospital potable water, but the serogroups and serotypes identified at contaminated sites inside the hospital did not match the *Legionella* in the infected patients. Later it was showed that aerosols from one or more cooling towers were the actual source of transmission. However, most patient care units did not have central mechanical ventilation or air conditioning. The hospital campus consisted of twenty-eight buildings. Twelve of the fifteen case patients occupied building 1. The four active cooling tower systems on the hospital grounds which were generally well operated and regularly treated with biocide were visibly fouled. One tower located on building 2, about one hundred feet upwind from building 1, was heavily contaminated with *L. pneumophila* which matched the serogroups and serotypes of nine infected patients. Smoke tracer studies showed that air entered building 1 through open windows on the south and west faces of the building, crossed the corridors and exited through open windows on the north and east faces of the building. Drift from the contaminated tower could easily be drawn into patients' rooms by this air movement (Garbe et al., 1985).

In a thirty-three patient outbreak of legionellosis in Glasgow in 1984, the likely source of infection was a cooling tower from which *L. pneumophila* serogroup 1 was isolated. Cases occurred downwind of this cooling tower up to a distance of 1700 meters (1.02 miles). Studies showed that the patients were more

likely to have a history of heart disease, and to sit by an open window (Baird et al., 1986).

Current opinion holds that the domestic hot water system in a hospital is the single most likely source of *Legionella*. Some studies found *Legionella* to exist in as many as 60% of hospital hot water systems sampled. It is likely that the 60% figure is a conservative assessment. Hospitals are a reservoir and amplifier of *Legionella*. Some factors involved in this dissemination are a susceptible host population, moderate water temperature and continuous recirculation in the design of their hot water system. The Joint Commission on Accreditation of Healthcare Organizations recommends a maximum water temperature of 120° F to prevent scalding, but this temperature is not hot enough to control bacterial growth. In fact *Legionella* bacteria multilply readily at this temperature. Little, if any, residual chlorine exists in hot water systems, while scale deposits and corrosion provide protective sites for *Legionella* growth. *Legionella* which enters the recirculated portion of the hot water system will be distributed throughout the entire system. Modifications and building reconstruction often produce capped off branch lines (dead ends) which create stagnant areas that accumulate nutrients, providing a breeding area for *Legionella* (Siwicki, 1989).

Phenomemon of Viable but Nonculturable Organisms

Since the 1800s microbiologists have cultured bacteria on solid agar surfaces. Through the years Robert Koch's technique of using culture for primary evaluation of a sample and gross microbial numbers has evolved into a

quantitative technique for microbial enumeration. Plate count numbers have been translated into "total bacterial count" or "total viable count". Theoretically, the number of microbes in a sample can be determined from the number of colonies growing on the surface of agar in a Petri dish, but plate count technique is often taught as a quantitative procedure without discussions of the limitations of the method. Culture has become the gold standard in microbiology, and the prevalent attitude is that if bacteria cannot form colonies, they must be dead (Buck, 1979; Mason et al., 1986).

Others, however, have questioned the validity of this criterion especially in aquatic work. Many microbiologists are realizing it is unrealistic to expect bacteria to shift their metabolism from survival in a highly stringent nutrient environment to rapid cell division and colony formation in a permissive nutrient rich environment on a solid agar surface. At a symposium on marine microbiology, R. D. Hamilton, a conference participant, commented, "... I think the plate count is the worst thing that ever happened to marine microbiology because it is so misused..." (Oppenheimer, 1968). It has been claimed that agar plate techniques enumerate only 1-10% of the viable organisms in soil, less than 0.1% in seawater and fail to detect as many as 90% of the coliforms in water (Mason et al., 1986).

One aspect of this problem regards the lack of a single universal medium on which all organisms can grow. Most organisms are sensitive to the type of growth medium used. Relatively luxuriant media nutritionally appear to shock cells,

while selective media on the other hand, impose intolerable levels of antibiotics and other exotic compounds. The pH of the media is a critical factor; different microbes require different pH values. Other causes for bacterial nonculturability include dormancy, inhibition by neighboring cells, inadequate incubation temperature and the fact that stressed or injured cells may have intrinsic difficulty in reproduction in all environments (Buck, 1979; Mason et al., 1986).

The detection of *Legionella* in water samples can be especially problematic. Water quality is as determined by analysis for coliforms. To be potable, water must contain less than one coliform per 100 ml (Boyd, 1988). *Escherichia coli* is the primary indicator of fecal contamination. However, *E. coli* fails to correlate with the presence of pathogenic bacteria , such as legionellas, *Vibrio cholera* and *Mycobacterium* (Young, 1996). There are no indicator organisms for *Legionella*; it must be directly detected (Hussong et al., 1987).

Because of the requirement for the direct detection of *Legionella*, culture is used for the isolation and enumeration of *Legionella* in water samples. If bacteria grow on buffered charcoal yeast extract agar (BCYE) but not on blood agar, the bacteria are presumptively identified as *Legionella*. However, cultural recovery of *Legionella* from environmental samples is hampered by the presence of faster growing bacteria (Toze et al., 1990). Most bacteria form visible size colonies in two days at 35° C, whereas *Legionella* takes 7-10 days. Often after outbreaks where *Legionella* was implicated no cultures can be recovered from environmental samples (Hussong et al., 1987). This led researchers to hypothesize that these

bacteria may be viable but nonculturable.

The fastidiousness of *Legionella* on solid culture media led several investigators to develop liquid media for the growth of this organism. A broth medium was formulated by Ristroph et al. (1980) at the U.S. Army Medical Research Institute of Infectious Disease in Maryland. *Legionella* had a lag phase of about eight hours in this medium. Log growth proceeded in eight to forty hours. The average generation time was ninety-nine minutes.

Culture is not the only method that can be utilized for the detection of *Legionella*. Direct fluorescent antibody techniques are applicable. Polyvalent fluorescent antibodies (PV) can be used to detect *Legionella pneumophila* serogroups 1-6 and also *Legionella micdadei*, the causative agent of Pittsburgh pneumonia (Myerowitz et al., 1979; Cordes et al., 1981). However, there are drawbacks to using PV antibodies. Although it is rare, cross reactivity with some nonLegionella bacteria can occur, and PV antibodies can bind to those cells whether they are living or dead. Alternatively, Genetic Systems Monoclonal Antibodies (GS) react with all serogroups of *L. pneumophila,* and there have been no reports of cross-reaction (Makin and Hart, 1989). However, there are reports that monoclonal antibodies failed to detect *L. pneumophila* in some environmental samples (Vickers et al., 1990). Monoclonal antibodies are expensive and, like polyvalent antibodies, bind to cells whether they are living or dead.

Because *Legionella* will be detected by the direct fluorescent antibody technique regardless of whether alive or dead, it becomes necessary to establish

techniques whereby the difference between living and dead bacteria can be assessed. Two assays currently are used to determine bacterial viability. The direct viable count method uses nalidixic acid, and the other assay uses the respiratory indicator, iodonitrotetrazolium chloride (INT). When viable bacteria are incubated with nalidixic acid and yeast extract, a direct viable count can be made of elongated cells. Nalidixic acid inhibits DNA synthesis thereby preventing cell division. The enlarged cells produced by growth without cell division are stained with acridine orange (Kogure et al., 1979; Byrd et al., 1991) or stained with fluorescent antibody instead of acridine orange for a species specific direct viable count (Rollins and Colwell, 1986). This technique, however, can only be used with certain gram negative bacteria. *Legionella* cells lyse when they are incubated with nalidixic acid (Hussong et al., 1987) making enumeration impossible.

The use of iodonitrotetrazolium chloride (INT) in combination with the fluorescent antibody can determine the viability of *Legionella* (Vesey et al., 1990). *Legionella* has an electron transport system as do most respiring bacteria. The oxidized form of the dye is placed in the water sample and taken up by actively respiring bacterial and algal cells of all types. The INT removes electrons at the ubiquinone-cytochrome b complex in the electron transport system to form the reduced compound INT-formazan (INT-F). The formazan will be deposited within the cell as dark red spots. Cells fluorescing with epifluorescent microscopy and have an INT-formazan crystal under bright-field microscopy are viable

Legionella cells. These cells can be viewed simultaneously under epifluorescence and bright-field illumination. However, if INT-F deposits are small, they may show weak contrast to the strong fluorescence of the surrounding area. In the absence of formazan deposits, the bacteria may still be viable but the state of activity may be below the level of detectability (Fliermans, 1985).

Bacterial Starvation Survival Strategies

The existence of a viable but nonculturable stage, in which bacterial cells are intact and alive but do not undergo cell division on conventional bacteriological media, appears to reflect a bacterial survival mechanism. Bacterial cells are in a dynamic state. Reduced respiration and low endogenous metabolism are mechanisms which enhance survival under limiting conditions. Bacteria that have the greatest capacity for longevity are small in size, by comparison to laboratory-grown strains. The small round forms of aquatic bacteria indicate that starvation is occurring, and starved cells more closely approximate the normal condition for bacteria than the overfed variety commonly seen under laboratory conditions (Roszak and Colwell, 1987).

Starvation-survival studies have been conducted on Ant-300, a marine *Vibrio*. When Ant-300 was inoculated into artificial seawater, its viability was maintained during the seventy week study period. Initially the cells decreased in size changing from rods to coccobacilli. In seven days endogenous respiration dropped to less than 1% of prestarvation levels yet these cells retained the capacity for active metabolism. When nutrients were added to the starvation medium, these

substrates were utilized (Novitsky and Morita, 1978; Amy and Morita, 1983.).

Roszak and Colwell (1987) have observed several genera of bacteria in various stages of survival and progressive dormancy as cells starve. Bacteria do not all have the same degree of viability but differ markedly from one another. Initially cells are culturable and recoverable in a favorable environment, but with nutrient limitation cells become smaller in size and their metabolism decreases. The cells become nonculturable, but they retain the ability to transport substrates. As starvation progresses the bacteria become dormant. They can no longer take up nutrients and are only recoverable through animal passage. Later the cells are still intact but cannot be recovered by animal passage. Eventually the cells are dead.

In an aquatic environment, the liquid expanse is bounded by interfaces with solids. The interfaces are sites where nutrients accumulate, allowing the microbes there to undergo active growth. Bacteria reaching a nutrient rich interface have a selective advantage in an otherwise nutrient-poor environment. In biofilm formation a conditioning film forms on a clean surface by the transport and adsorption of organic materials from the bulk water. Next bacteria colonize the conditioned surface. At first there is reversible adsorption, i.e. bacteria attach to the surface for a period of time but then re-enter the bulk water perhaps because of the shear force of the fluid flow. Bacteria which do not desorb after a period of time become more permanently attached to the surface. Extracellular polymers produced by the cells hold the biofilm together. Microbial cells in the biofilm may

grow and multiply using the organics in the biofilm and the substances which are floating by in the bulk water as nutrients. The biofilm continues to grow as more extracellular polymers form and as more cells and particles attach and detach (Characklis and Marshall, 1990).

There are times when entire sections of a biofilm may be sloughed off of the surface due to mechanical disturbances. There have been reports that link external construction to increased bacterial activity in domestic water systems. Mechanical vibration of undergound water mains may result in the release of bacteria-containing scale from pipe surfaces (Wireman et al., 1992).

Members of the genus *Pseudomonas* are common members of biofilms in aqueous environments. They frequently serve as a primary colonizer of inert surfaces in such environments. Certain species of *Pseudomonas* are known to be active producers of extracellular polysaccharides (Page and Gaylarde, 1990) and therefore of utmost importance in biofilm formation. The polysaccharide cements *Pseudomonas* to the inert surface as well as helping to trap other species of microorganisms.

As several species of bacteria colonize the same surface a microbial community is established. The cells grow embedded in their own organic polymer matrix as biofilms. In natural and industrial environments, 99.9% of all bacteria are in biofilms (Bodine, 1993). Channel structures within biofilms facilitate the transport of vital nutrients throughout the community, and steep solute gradients within the inner layers of matrix affect molecular diffusion. The resilience of

microbial biofilm communities against adverse conditions and toxic compounds exemplifies the competitive advantage this life form confers. The stability of such systems is largely attributed to the interdependency of biofilm community members and their ability to create favorable microenvironmental conditions compared with those in the nearby liquid phase (Massol-Deya et al., 1994).

Cells in biofilms are difficult to kill. Infections often result from biofilms forming on medical devices such as artificial joints, heart valves and catheters. Antibiotics will kill off any planktonic organisms that might be causing fever and pain, but as soon as antibiotic therapy is suspended, the infection reappears because the cells in the biofilm weren't killed (Bodine, 1993).

Legionella pneumophila forms biofilms as part of its survival strategy. Some *L. pneumophila* survive the disinfection process and enter building water systems and colonize pipe surfaces. In buildings where cold water is subject to stagnation and warming (20-30° C) and especially in the hot water system (30-60° C) there is extensive colonization of surfaces and, perhaps, multiplication in the water itself. Contaminated hot water tanks were thought to be the primary source of the organism, but even when tanks are sterilized, *L. pneumophila* persists in the periphery of the hot water system. Faucet fittings, mixer taps and shower fixtures provide an ecological niche for *L. pneumophila* and make the organism available for aerosolization when faucets are turned on (Colbourne et al., 1984; Wright, et al., 1989).

Numerous studies have been conducted which show that rubbers and their

constituents can affect the growth of *L. pneumophila* in water. Following a 1980 outbreak of Legionnaires' disease at Kingston Hospital in London, chlorination and an increase in the hot water temperature failed to decontaminate fittings at the periphery of the plumbing system. *L. pneumophila* was isolated from rubber washers, and laboratory experiments demonstrated growth of this organism in tap water in contact with these washers (Colbourne et al., 1984). Niedeveld et al. (1986) demonstrated that nine of fourteen types of rubbers tested enhanced growth of *L. pneumophila* in potable water as did fifteen of thirty rubber constituents, including stearic acid and ferric oxide. However, the use of certain ingredients in the manufacture of rubber will not indicate if the finished product will support the growth of aquatic bacteria. Vulcanization causes chemical changes so the finished product needs to be tested to see if it affects water quality (Colbourne and Ashworth, 1986). Wireman et al. (1993) compared eight different surface types for *L. pneumophila* attachment and found that ethylene propylene diene (EPDM) rubber was consistently superior to other surfaces in the quantitative attachment of legionellae. Incubation of ethylene-propylene rubber in water for three days produced elevated levels of total organic carbon. The extensive biofilm growth of *L. pneumophila* on this surface was attributed to the leaching of nutrient from the rubber (Rogers et al., 1994).

Virulence of *Legionella pneumophila*

The mere presence of legionellae in water does not necessarily mean they are capable of causing disease. The organisms can exist as either virulent or

avirulent forms and are capable of interchanging between the two states. Virulence is most commonly determined by inoculating *L. pneumophila* into either guinea pigs or embryonated chick eggs and determining death rates at varying concentrations of inocula.

McDade and Shepard (1979) demonstrated that when *L. pneumophila,* isolated from guinea pig spleen or embryonated eggs, was cultured on Mueller-Hinton (M-H) or Feeley-Gorman (F-G) agar, there was a change in virulence. Experiments showed the *L. pneumophila* lost virulence after ten passages on either M-H or F-G agar. The organisms passed on F-G agar were at least 100-fold less lethal for eggs than organisms passed on M-H agar.

Elliott and Johnson (1982) showed that when virulent *L. pneumophila* is passaged on M-H agar, it remains virulent for guinea pigs and embryonated eggs for two passages. By the fifth passage the cultures become avirulent for guinea pigs. Charcoal yeast extract agar was the best medium for maintaining the virulence and flagellation of *L. pneumophila*; twelve passages *did not* result in a reduction of virulence or loss of flagella. When an avirulent form was passed through guinea pigs six times, a virulent flagellated form was recovered. When avirulent *L. pneumophila* was passaged twelve times in embryonated eggs, a nonflagellated form with an increased virulence was recovered.

Research shows that buffered charcoal yeast extract (BCYE) agar is effective in maintaining the virulence of *L.pneumophila.* Yamamoto et al., (1993) showed that the virulence of *L. pneumophila* was maintained using a single colony

transfer for each passage on BCYE agar. After thirty passages no loss of virulence occurred in comparison to nonpassaged parental isolates.

Studies show that heat shock affects the avirulent to virulent conversion in *L. pneumophila*. Samples heat treated at 45° C for either ten or thirty minutes resulted in a change of the subgoups of some of the *Legionella*. *L. pneumophila* serogoup 1 Olda subtype which is rarely associated with disease but is widely found in potable water converted into Bellingham subtype, a more virulent subtype. The experiments suggested that virulence could be altered by heat shock (Colbourne et al., 1988; Watkins et al., 1985). When an avirulent strain found in cold water passes into a hot water system or cooling tower and encounters higher temperatures (30-60° C), heat shock may initiate changes in virulence. This would be a plausible explanation why warm water systems are associated with *Legionella* outbreaks (Colbourne and Dennis, 1989).

Mauchline et al. (1994) further demonstrated the relationship between temperature and virulence. The virulence of two strains of *Legionella pneumophila* was shown to be significantly reduced when the culture temperature was lowered from 37 to 24° C; when the temperature was returned to 37° C, virulence increased. When temperatures are measured in cooling towers, they are most commonly taken in the pond which is the coolest part of the system. Because temperature reversibly modulates virulence, the temperature found at the condenser of the unit, which is usually between 35 and 45° C, could induce

virulence in *L. pneumophila* and increase its chance for causing disease in humans.

Studies of *L. pnemophila* virulence led to the discovery of the *mip* (macrophage infectivity potentiator) gene. The Mip protein is a membrane associated protein of 24 kDa which is distinct from the major outer membrane protein (Engleberg et al., 1989). Mip-negative mutants of *L. pneumophila* have a reduced ability to invade eukaryotic cells by a factor of 100 when compared to Mip-positive cells (Cianciotto et al., 1989). The *mip* gene of *L. pneumophila* exhibits homology with *mip*-like genes of other *Legionella* species (Bangsborg et al., 1991; Cianciotto et al., 1990). Genes which show about a 50% homology to the *L. pneumophia mip* gene are found in other pathogenic bacteria like *Chlamydia trachomatis*, *Neisseria meningitidis* and *Pseudomonas aeruginosa* suggesting a common mechanism in bacterial pathogenicity (Hacker et al., 1993).

The major outer membrane protein (MOMP) of *L. pneumophila* also plays a role in virulence. The MOMP protein binds the C3 factor of complement. This mediates the macrophageal engulfment of *Legionella* (Bellinger-Kawahara and Horwitz, 1990).

Avirulent variants differ from virulent organisms in numerous aspects. Morphologically avirulent strains tend to display an elongated cell shape (Nowicki et al., 1987). They have a reduced survival in aerosols (Dennis and Lee, 1988), and the capability to replicate intracellularly in monocytes, macrophages and protozoa is lost (Hacker et al., 1992).

Hussong et al. (1987) inoculated embryonated eggs with microcosm water

samples of *L. pneumophila*. Suspensions were made whereby each egg received an average of less than one culturable bacterium (0.6 *L.p.*/ml). Lethal infections occurred in these eggs. Culture of the yolk sac tissue from these embryos resulted in dense growth of *L. pneumophila*. Also *L. pneumophila* in the yolk sacs was observed by fluorescent microscopy. The researchers concluded that previously nonculturable bacteria had become culturable following infection of chick embryos.

There are drawbacks to using guinea pig and embryonated egg inoculation for determining the virulence of *Legionella*. Guinea pigs are relatively unsusceptible to most bacteria, and an inoculum of 10^6 cells is required. The need for such a large inoculum may indicate the method is not sufficiently sensitive for detection of subtle changes (Hussong et al., 1987; McDade and Shepard, 1979).

In nature *L. pneumophila* is present with an extensive number of other bacteria. NonLegionella bacteria are also capable of using embryonated egg nutrients, and the slow growing *Legionella* are no match for the other aquatic organisms that have faster generation times and are initially in greater concentration (Fliermans, 1985).

Growth Chambers and Water Systems

Researchers often construct growth chambers or water systems to aid in the study of *L. pneumophila*. Berg et al. (1985) used a chemostat to test the effectiveness of chorine dioxide against *L. pneumophila* grown in continuous culture versus batch culture. A pure culture of this organism was grown in a

chemostat under different nutrient concentrations and temperatures. The more slowly grown cells from the chemostat were about two orders of magnitude more resistant to chlorine dioxide than batch grown populations.

Schofield and Wright (1984) and Schofield and Locci (1985) constructed a water system in which colonization of rubber and metals commonly found in a hot water distribution system could be examined. A water bath housed a one liter flask of a starter inoculum of *L. pneumophila* which had been cultured on artificial media a maximum of three times. The bacteria were introduced into an enclosed system filled with 500 ml of unsterilized water from a domestic hot water supply. Water and inoculum were pumped into three stoppered glass chambers each containing a different substance – twists of copper, twists of stainless steel and glass beads plus aluminum discs. After passing through the glass chambers the fluid was combined and recirculated to the flask. All tubing in the system was silicone. The fluid in the system was circulated intermittently, drained out each week, and replaced with fresh warm tap water. Water samples were taken periodically from the system and evaluated for the presence of *L. pneumophila* by culture and fluorescein labeled antibodies. After ten weeks the system was drained and dismantled. Various components of the system were scraped with a sterile scalpel. *L. pneumophila* counts were highest from the rubber stoppers and lowest from the copper turnings.

Muraca et al. (1987) constructed a model plumbing system for assessing the effectiveness of chlorine, heat, ozone and UV light against a cultured

environmental isolate of *L. pneumophila*. The system utilized a centrifugal pump to recirculate thirty-eight liters of nonsterile tap water through copper tubing. Flow rate was adjusted to 3.0 liters/min and monitored with a flow meter. Disinfectants were tested individually. Chlorine was from a reservoir; heat was regulated by a hot-water tank thermostat; ozone was introduced by an injector; UV energy was generated within a flow-through UV chamber. A suspension of *L. pneumophila* was added to the system and allowed to circulate for approximately 45 to 60 min before the disinfectant was applied. Water samples were collected from sample ports and evaluated by culture. All four disinfectant methods were effective in killing *L.pneumophila.*

Haldane et al. (1993) at Victoria General Hospital in Nova Scotia constructed a recirculating hot water system composed of the same materials found in their hospital which was contaminated with *L. pneumophila* for more than eight years. The system was constructed with a loop of copper piping and contained a twelve imperial gallon hot water tank. Circulation of water was constant and maintained by a circulating pump. Temperature and pressure were monitored by gauges connected to the loop. There were three faucets – one draining directly from the loop and one faucet draining each dead end. Inocula were introduced by an injection pump connected to an injection port. The model was primed with tap water and inoculated with a clinical isolate of *L. pneumophila* grown on BCYE. The first sample from the system was usually removed within twenty-four hours of inoculation and cultured. The system was drained and

reinoculated on five different occasions. A different water temperature (from 35-50°C) was maintained during each inoculation. *L. pneumophila* was never recovered on BCYE despite the large inocula used in the system. A number of possible explanations for this apparent failure of colonization were: metal ion concentrations, lack of dissolved oxygen or nutrients, a high pH, only small areas of nonmetallic surfaces that could be colonized (legionellae colonize copper and brass slowly; older systems may favor colonization by deposition of scale that would cover the less favorable metal surface), presence of nonLegionella bacteria which don't support growth of legionellae and conversion of the organisms to a nonculturable state.

The purpose of this project was to explore the viable nonculturable phenomenon with *L. pneumophila*. This study sought to determine answers to the following questions:

1. Using a model water system that mimics a recirculating hospital hot water system, can viable planktonic and biofilm populations of *L. pneumophila* be maintained?
2. Can the viable nonculturable phenomenon be demonstrated for planktonic and biofilm populations of *L. pneumophila*?
3. Do nonLegionella bacteria influence the survival of *L. pneumophila* or the conversion from or to the viable nonculturable state?
4. Can virulence of *L. pneumophila*, as measured in embryonated chicken eggs, be used to examine the relationship between viability and virulence?

5. Can a comparison of the characteristics of planktonic and biofilm populations of *L. pneumophila* be used to determine if there are critical differences?

MATERIALS AND METHODS

Construction of a Model Hot Water System

The model system used here (Figure 1) was constructed of a closed loop of one half inch cPVC pipe. There were two dead ends where stagnation of water could occur creating sites for biofilm formation and bacterial growth. The six gallon hot water tank maintained a temperature of 110-120° F (43-49° C). The water was circulated by a Teel bronze body, 1/26 horsepower, in-line circulating pump rated for 230° F at a maximum pressure of 140 psi. Temperature and pressure were monitored by gauges connected to the loop. A bleed valve at the top of the loop was used to help eliminate air pockets trapped in the system. Flow rate was determined by a flow meter; a flow rate of 2.4 gal/min was maintained at 0 psi. There were three sampling ports, one for each of the two dead ends and one for the main loop. Ethylene propylene diene methylene (EPDM) rubber coupons, measuring 3 inches by 1 inch, were inserted into four coupon holders, one holder in each dead end and two holders in the main loop. When it was necessary to add tap water to the system, the water was run through a charcoal filter to remove chlorine and organics.

When the system was first built in April, 1995, it was filled with water from multiple sites in a health care facility in the Detroit area that were known to be contaminated with *Legionella pneumophila*. No more than 500 ml of water taken first in the flow from the hot water tap was collected from any one site per day.

Legionella is primarily found in the hot water lines, and the first off water contains the highest counts (Colbourne et al., 1884). Before hospital water was added to the system, an aliquot was cultured on BCYE agar and 100 ml passed through a 0.2 micron polycarbonate filter for polyvalent staining to confirm the presence of *Legionella.* After months of operation and refinement to the system, Genetic Systems monoclonal antibodies were used to stain a sample from the water system. The organisms in the system were polyvalent positive but Genetic Systems negative. This situation could be due to: (i) the presence of *Legionella micdadei* instead of *Legionella pneumophila,* (ii) cross reaction of the polyvalent with nonLegionella bacteria, or (iii) the failure of Genetics Systems to detect *L. pneumophia* in the system. To rectify the problem the system was chlorinated, drained and flushed numerous times. DPD tablets were used to check for the presence of residual chlorine.

In January, 1996 the system was filled with tap water run through the charcoal filter and inoculated through the bleed valve with a one time cultured sample of *L. pneumophila.* The *L. pneumophila* was isolated in pure culture by plating hospital water on BCYE agar. Cells were PV + and GS +. Although the system was not specifically seeded with bacteria other than *L. pneumophila,* a population of these organisms developed; the water system then contained a mixed bacterial population.

Procedure for Polyvalent Staining on a Filter

The quantification of *L. pneumophila* in a water sample was achieved using

the following procedure developed at Biological Research Solutions Inc., Detroit, Michigan (unpublished results).

1. Fix 100 ml of a water sample with 10 ml of 11% neutral formalin (approximately 1% final concentration).

2. Filter the fixed sample through a 0.2 micron polycarbonate filter using a vacuum filtration system. The cells remain on the smooth upper surface of the filter.

3. Stain the surface of the filter with 1 drop of polyvalent fluorescent antibodies and allow them to remain for 20 minutes.

4. Rinse the antibodies off the polycarbonate filter by drawing sterile phosphate buffered saline through the filter using the vacuum filtration system.

5. Allow the polycarbonate filter to dry and mount the stained portion of the filter on a microscope slide.

6. View the slide using epifluorescent microscopy and count the number of fluorescing *Legionella* cells in 25 fields.

7. The conversion from cells per 25 fields to cells per ml was determined by calibration with *L. pneumophila*, Philadelphia strain. The number of *Legionella* cells in 25 fields times 14 will equal the number of *Legionella*/ml in the original water sample.

Identification of nonLegionella Bacteria

Three species of nonLegionella bacteria from the water system were isolated on Plate Count Agar (Difco , Detroit, Michigan). From the hospital water sample two culturable species of nonLegionella bacteria on plate count agar were found. All of these organisms were Gram negative. The API 20 Enteric system (Analytab Products, Inc. purchased from bioMerieux Vitek, Inc., Hazelwood, MO) was used for their identification. Used in conjunction with the API Profile Recognition System, it enables laboratory personnel to identify members of the

family Enterobacteriaceae and other Gram-negative bacteria. In addition to identifying these five nonLegionella organisms, they were stained with polyvalent fluorescent antibodies to test for cross-reactivity.

Effect of nonLegionella Bacteria on *Legionella pneumophila* Culturability

In order to determine if the nonLegionella (nonL.p.) bacteria influenced the culturability of *Legionella pneumophila* (*L.p.*), the following experiment was performed. Two sterile bottles were each filled with 100 ml of sterile filtered hospital water. To bottle #1 and #2 an estimated 100 *L.p.* / ml, isolated by culture from hospital water, were added. In addition, approximately 100 one time cultured hospital nonL.p./ml were added to bottle #2. The bottles were incubated at 35°C. Every week an aliquot was removed from each bottle and plated on BCYE and plate count agar. After a one month period, the number of *L. pneumophila* in each bottle was determined by polyvalent staining.

INT Assay for Determination of Bacterial Viability

A sample can be evaluated for *Legionella pneumophila* viability using the iodonitrotetrazolium chloride (INT) assay (Zimmermann et al., 1978). The INT assay can be used to detect microorganisms which are viable but nonculturable. If a microbe has an active electron transport system, INT will remove hydrogen atoms and be reduced to a red deposit of INT-formazan.

Modifications have been made by researchers to the Zimmermann method. When using the INT assay with *L. pneumophila*, the sample and INT should be incubated for sixty minutes (Fliermans et al., 1981). The pH of the INT should be

adjusted to 9.6 (Fliermans et al., 1982).

In this project yeast extract broth was utilized in the INT assay in order to resuscitate stressed cells with low rates of respiration such as the *L. pneumophila* in the water system. Increased electron transport activity results in the formation of larger, more readily detectable INT-F deposits.

1. Collect water sample in a sterile container.
2. Put 50 ml water sample, 50 ml double strength yeast extract broth and 10 ml 0.2% INT pHed to 9.6 into a sterile bottle.
3. Mix carefully.
4. Incubate in the dark at 35° C for 5 hours.
5. Add 10 ml of 11% neutral formalin (approximately 1% final concentration).
6. Filter the entire contents of the bottle through a 0.2 micron polycarbonate filter.
7. Stain a portion of the filter with polyvalent fluorescent antibody.
8. Mount the stained area of the filter on a microscope slide.
9. Using mixed light examine the filter microscopically for formazan spots in fluorescing cells.

Pure *Legionella pneumophila* INT Bottle Experiment

Due to the rapid growth of the nonLegionella bacteria in yeast extract broth and their formation of large amounts of INT-formazan, filtration of the entire INT incubated water sample was difficult to achieve. Consequently, pure *L. pneumophila* biofilms were grown outside of the water system. For this an EPDM coupon was inserted into each of two sterile bottles containing 100 ml sterile filtered water from the water system and 1 ml of a suspension of one time cultured

hospital *L. pneumophila* containing 3 X 10^6 colony forming units/ml. The bottles were incubated for one month at 35° C after which INT-formazan formation of the biofilm and planktonic cells was measured.

Procedure for Inoculation and Recovery of *Legionella pneumophila* in Embryonated Eggs

The virulence of *Legionella pneumophila* is determined by injecting cells into embryonated chick eggs. The protocol used in this study was a modification of the method outlined in the Center for Disease Control Laboratory Manual (McDade, 1978).

1. Embryonated hen eggs that had been incubated for one week at 37°C were used. The eggs were candled prior to inoculation to insure that they were healthy.

2. Three ml of inoculum were drawn into a syringe fitted with a 20 gauge 1-1 ½" needle. The needle was covered with the shield and the filled syringe placed on the bench top.

3. The top of the eggs were disinfected with 70% ethanol. The disinfectant was allowed to air dry. An egg punch was disinfected by dipping it in 70% alcohol and passing it through a flame. A hole was punched in the top of each egg and then each egg was inoculated with 0.5 ml of a water sample, being sure the needle was inserted completely. Each egg was sealed with Duco cement. The eggs were incubated at 37° C for seven days.

4. After incubation each egg was broken into a sterile petri dish and the age of the embryo determined. Eggs that died on Days 7, 8, or 9 were discarded (nonspecific death). The yolk sac membranes of embryos which died Day 10 or later were removed with sterile forceps and put into individual screw cap test tubes containing sterile deionized water.

5. Tubes containing the yolk sacs membranes were vortexed to suspend *L. pneumophila* growing on the membranes. Dilutions were made of each tube and plated on BCYE agar and plate count agar. The plates were incubated for 7-10 days.

6. Suspensions were made of BCYE colonies showing typical *L.*

pneumophila morphology. Confirmation of *L. pneumophila* isolates was made with polyvalent fluorescent antibody staining.

Calculation of the Lethal Dose $_{50}$

The number of microbes which causes death in 50% of embryos inoculated can be quantified. This is expressed as the lethal dose (LD_{50}). The LD_{50} is based on statistical considerations and is widely used in measuring virulence of organisms. The method used is as follows:

1. Tenfold dilutions of *Legionella pneumophila* (*L.p.*) were prepared.
2. A ½ ml aliquot of each dilution was used to inoculate embryonated eggs.
3. The dilution at which 50% of the embryos died was extrapolated. When the LD_{50} falls between two dilutions, the 50 percent point can be calculated using a formula for proportional distance devised by Reed and Muench (1938).
   $$\frac{\text{50 percent - (mortality at dilution next below)}}{\text{(mortality next above) - (mortality next below)}} = \text{proportional dist.}$$
4. The difference between the inoculum next above and next below the 50 % mortality was determined.
5. The difference was multiplied by the proportional distance and the product added to the lower inoculum.
6. The sum was divided by 2 because only ½ ml aliquots were inoculated into the eggs.

A sample calculation of an LD_{50} follows:

Water System (Planktonic Cells) Date of inoculation 2-7-97

# Eggs inoculated minus nonspecific death	Inoculum (*L.p.*/ml)	#Embryos dead day 10 or later	Mortality rate
8 – 1 = 7	1.2	5	71.4%
7 – 1 = 6	0.12	1	16.7%

[Step 3] 50% - 16.7% / 71.4% - 16.7% = 33.3/54.7 = 0.61(proportional distance)

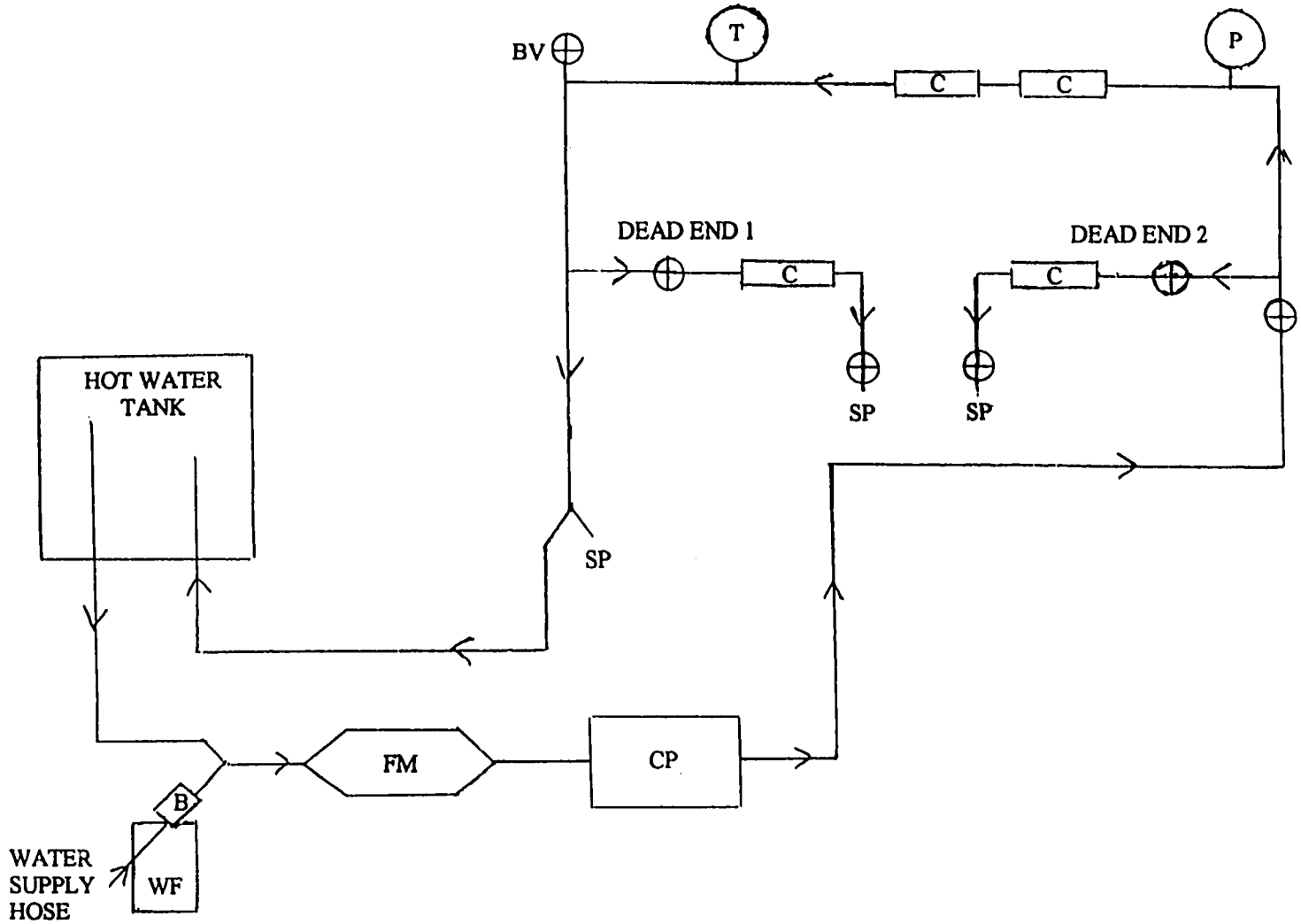
[Step 4] 1.2 – 0.12 = 1.08

[Step 5] 1.08 x 0.61 (proportional distance) = 0.66

0.12 + 0.66 = 0.78

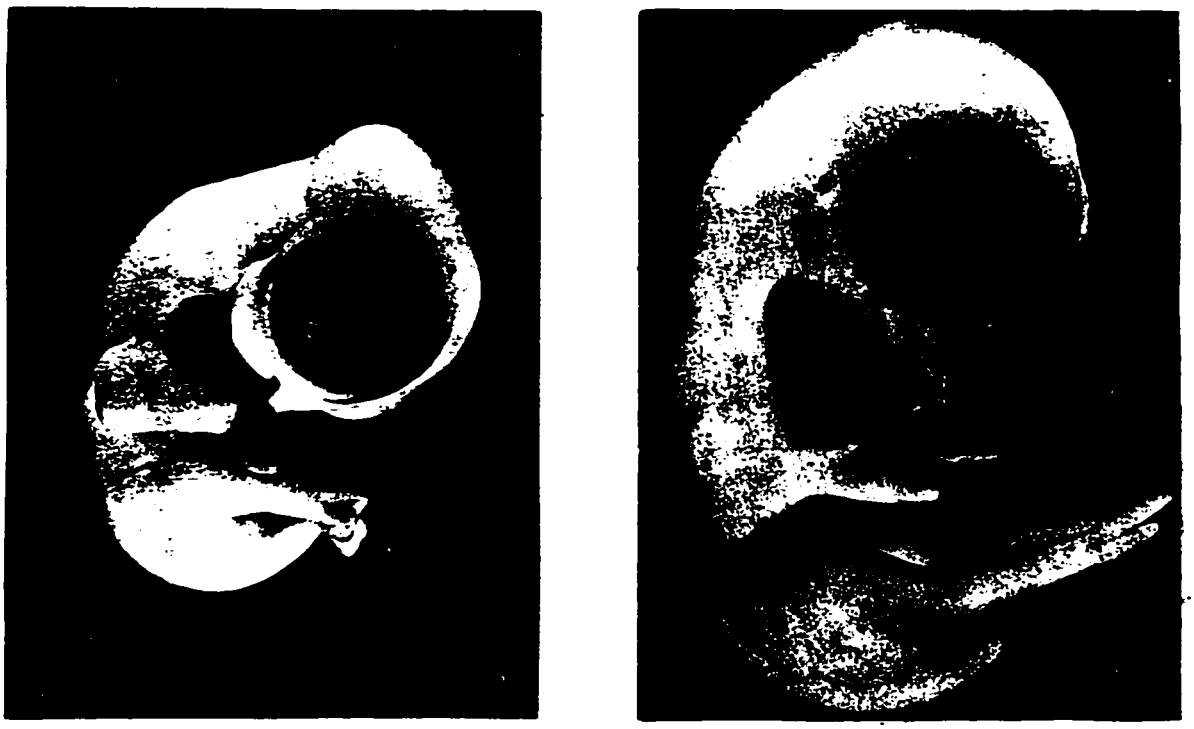
[Step 6] 0.78 / 2 = 0.39 (LD_{50})

Figure 1. Schematic Diagram of Recirculating Water System



B, back flow preventer; BV, bleeding valve; C, coupon holder; CP, circulating pump; FM, flow meter; P, pressure gauge; SP, sampling port; T, temperature probe; WF, charcoal water filter

Figure 2. Nonspecific Death of Chicken Embryos



Day 7 Day 8-9

Chicken embryos that died on days 7-9 were considered as nonspecific deaths. Photographs of seven and eight to nine day embryos are shown. (From Hamburger, V. and Hamilton, H.L., *J. Morph.* 88:85,87. 1951. With permission.)

RESULTS

Culturability of Water System *Legionella pneumophila*

A recirculating hot water system was constructed to simulate environmental conditions found in health care facilities. During its two years of operation, the model water system was inoculated on seven occasions with one time cultured *Legionella pneumophila* cells from a hospital water sample. The culturable cells added to the system were positive by both polyvalent (PV) and Genetic Systems (GS) fluorescent antibody staining. Biofilm and planktonic *L. pneumophila* cells in the system were monitored microscopically using fluorescent staining and by culture on BCYE agar plates to determine if cells would become nonculturable yet still be detectable by staining.

L. pneumophila readily attaches to surfaces especially to substances such as rubber. EPDM rubber coupons were inserted into the system to allow *L. pneumophila* to attach and thus serve as a source of biofilm cells. Cells were scraped with a razor blade from coupons which had been in the system for at least a month. PV and GS staining indicated *L. pneumophila* were present in biofilms, but they were never recoverable by culture on BCYE agar. *L. pneumophila* biofilm cells were consistently nonculturable.

The water system was also monitored for the number of planktonic *L. pneumophila* cells using polyvalent staining and culture techniques. Although PV cell counts dropped after each inoculation of the system (Tables 1-7 and figures 3-9), planktonic *L. pneumophila* were still detectable by staining five months after

inoculation (Table 6 and figure 8). However, planktonic *L. pneumophila* became nonculturable within a few days after inoculation.

To determine if the drop in the stained cell count after inoculation of the system was due in part to biofilm formation, a coupon was inserted into the water system at the time of an inoculation. Three days later the coupon was removed and stained directly on the rubber surface with PV stain. Eleven *L. pneumophila*/10 fields of view were counted microscopically indicating that some of the *L. pneumophila* added to the system were attaching to surfaces.

To further demonstrate that biofilm and planktonic *L. pneumophila* became nonculturable in the water system, a selective medium used to isolate *Legionella* species was used. Cells were also plated on DGVP, a buffered charcoal yeast extract agar containing antibiotics. No culturable *L. pneumophila* were recovered on this medium.

In another attempt to recover planktonic *L. pneumophila* by culture, a 25 ml water sample was taken from the system and passed through a 0.2 micron polycarbonate filter. The filter was then directly placed on BCYE agar. No culturable *L. pneumophila* were recovered.

After each of seven inoculations of the water system, viable *L. pneumophila* became nonculturable within a few days after inoculation but were still detectable by fluorescent staining. This was true for both biofilm and planktonic cells. Subsequent research reported below will provide evidence that these nonculturable cells remained viable.

Identification of nonLegionella Bacteria

Legionella pneumophila were not the only bacteria present in the water system. Although the system was not seeded with nonLegionella organisms, the system was not sterile and a mixed bacterial population developed. The API 20E and API Profile Recognition Systems were used for identification of the nonLegionella.

Three species of bacteria isolated from the water system and two species isolated from the hospital water sample used as the source of *L. pneumophila* cells were identified. The water system organisms were designated as WSB, WSP and WSY; hospital bacteria were designated 4CP and 4CY. WSB was identified by the API Systems as *Pseudomonas* sp., *Alcaligenes* sp. or *Bordetella bronchiseptica* with a rating of good likelihood but low selectivity. This meant it was quite certain that WSB belonged to one of the three genera listed but specifically which genus or species within that genus could not be determined. WSP was also designated with good likelihood but low selectivity as *Alcaligenes* sp., *Pseudomonas* sp., CDC Group IV C-2 or *Achromobacter xylosoxidans*. Because WSY produced yellow pigmented colonies, there was very good identification as *Pseudomonas paucimobilis*. 4CP was identified with good likelihood but low selectivity as *Pseudomonas* sp., *Alcaligenes* sp. or *Bordetella bronchiseptica*. 4CY also produced yellow colonies, and there was very good identification that the organism was *Pseudomonas paucimobilis*.

From these results it was shown that WSY and 4CY, an organism from the

water system and an organism from the hospital water sample, were the same species of bacteria. It could not be conclusively shown whether or not 4CP, the second species isolated in the hospital water, was also found in the water system. The five isolated nonLegionella bacteria did not cross-react with the polyvalent fluorescent antibodies used in this research project.

Effect of nonLegionella Bacteria on *Legionella pneumophila* Culturability

An experiment was performed to determine if nonLegionella bacteria played a role in the culturability of *Legionella pneumophila* . Two autoclaved bottles each contained equal amounts of sterile filtered hospital water and *L. pneumophila* cells. To bottle #2 nonLegionella cells were also added. The bottles were incubated at 35°C. Table 8 indicates that the weekly culturing of aliquots from each bottle on BCYE agar showed a decline in *L. pneumophila* culturability in both bottles. *L. pneumophila* became nonculturable whether nonLegionella were present or not. Furthermore, the conversion from culturable to nonculturable proceeded at the same rate in both bottles. After a month of incubation, polyvalent counts of the number of *L. pneumophila* present in each of the two bottles were approximately the same. It was also observed that within a week after inoculation the nonLegionella bacteria in bottle #2 had multiplied and reached high levels.

INT Assay for Determination of Bacterial Viability

Although *Legionella pneumophila* cells were visible microscopically using fluorescent staining, this did not indicate whether the cells were living or dead. Iodonitrotetrazolium chloride (INT) was used to determine the viability of *L.*

pneumophila cells. Cells with a functional electron transport system convert INT into INT-formazan (INT-F) which is seen microscopically as red insoluble deposits in cells. Formazan deposits, observed in cells which also fluoresce with polyvalent stain, indicate viable *L. pneumophila*. Yeast extract broth (YEB) was used in the INT assay to resuscitate cells which may be stressed.

A control with formalin killed cells was used in the INT assay. Yeast extract broth, water system planktonic cells and formalin were incubated with INT for five hours. At the end of the incubation period, microscopic examination of thirty fluorescing formalin killed *L. pneumophila* cells showed that none of these cells contained INT-formazan deposits. Dead cells do not have a functioning electron transport system and therefore should not contain INT-F.

Table 9 and figure 10 show that when one time freshly cultured hospital *L. pneumophila* cells were incubated with YEB and INT for 1 hour or 2.5 hours, the majority of the cells contained INT-F deposits, 92% and 88% respectively. Time had little effect on the percentage of cells that formed INT-F, but formazan deposits were larger and therefore easier to detect with the longer incubation period.

Table 10 and figure 11 show the percent of water system planktonic and biofilm *L. pneumophila* cells with INT-F formation with YEB and INT. A longer incubation period for planktonic cells, 5 hours instead of 3 hours, did not increase the percentage of cells which formed formazan. Because of the more favorable environment existing within a biofilm, as compared to the bulk water, I expected

biofilm cells to be more metabolically active and have a greater respiratory rate than planktonic cells. The results indicated, however, that neither biofilm nor planktonic *L. pneumophila* cells had consistently greater respiratory rates. More importantly, the INT assay conclusively proved the viable nonculturable pnenomenon. Although planktonic and biofilm cells in the water system were nonculturable, a percentage of these cells were viable with functioning electron transport systems.

In some cases the presence of rapidly multiplying nonLegionella bacteria made it difficult to filter the entire contents of the INT incubation bottle due to clogging of the polycarbonate filter. To alleviate this problem, pure *L. pneumophila* biofilms were grown on EPDM coupons inserted into bottles containing sterile filtered water from the water system and pure one time cultured hospital *L. pneumophila* cells. Table 11 and figure 12 show that some of the biofilm cells were viable under these experimental conditions, but the planktonic cells were not.

Viability results can be summarized as follows. One time cultured hospital *L. pneumophila* cells have the greatest respiratory rate of all the cell types assayed. The length of time cells were incubated with INT did not affect the percentage of cells which formed INT-formazan. However, the longer incubation period made the formazan deposits easier to detect. No significant difference between the respiratory rates of water system planktonic and biofilm *L. pneumophila* cells was shown, but each sample had a percentage of cells which were viable but

nonculturable.

Virulence Determination of Water System Organisms

This segment of the research sought to determine whether the virulence of *Legionella pneumophila* related to the viable nonculturable state. Virulence can be determined by inoculation of embryonated chick eggs that have been incubated for seven days. Embryos that die on days 7-9 are considered nonspecific deaths. The number of embryos which die on day 10 or later is used in the calculation of the lethal dose $_{50}$ (LD_{50}), the dose of microorganisms that is lethal for 50% of the hosts. The proportional distance formula in the Reed and Muench method was used for calculation of LD_{50s} where the fifty percent point lies between two doses.

The LD_{50} for each different cell type was determined three times, and the mean for the three trials calculated. Table 12 shows data from the three trials where one time cultured hospital *L. pneumophila* cells were inoculated into embryonated eggs, and in table 13 this data is utilized for the determination of the three LD_{50s}. Table 14 lists data collected by inoculating one time cultured nonLegionella bacteria from the water system into embryonated eggs followed by a table that includes the LD_{50s} from these inoculations. Tables 16 and 17 provide experimental results used in the calculation of LD_{50s} for water system planktonic cells. The effect water system biofilm cells had on embryonated eggs and the three LD_{50s} are listed in tables 18 and 19.

Table 20 shows the mean LD_{50s} for cultured hospital *L.pneumophila* cells, nonLegionella cells and water system planktonic and biofilms cells. Analysis of

table 20 indicates the cultured hospital *L. pneumophila* cells were by far the least virulent of all the cell types studied. An average of 26.1 cultured *L. pneumophila* cells were needed to kill half of the embryos. The planktonic nonLegionella which developed in the water system were more virulent. It only took an average of 2.37 nonLegionella cells to kill half of the embryos inoculated.

Table 20 also indicates planktonic cells were more virulent than biofilm cells. However, water system planktonic and biofilm cells were a mixture of *L. pneumophila* and nonLegionella bacteria. It took an average of 0.89 *L. pneumophila* and 2.25 nonLegionella planktonic cells to kill half the embryos; it took an average of 10.3 *L. pneumophila* and 6.03 nonLegionella biofilm cells to elicit the same effect.

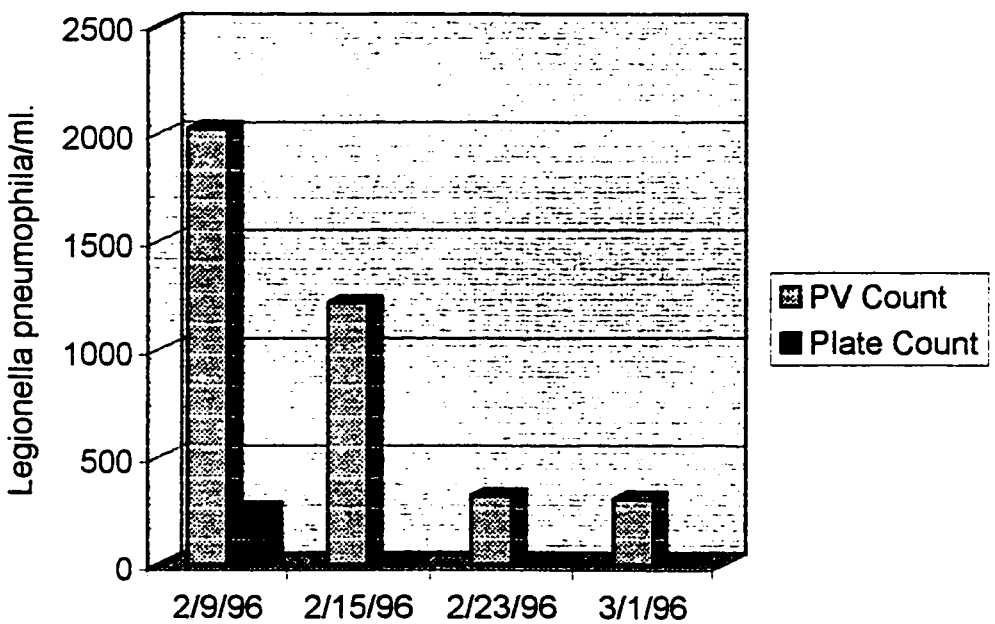
The mean LD_{50} for cultured planktonic nonLegionella from the water system was 2.37. When water samples containing both *L. pneumophila* and nonLegionella were inoculated into eggs, an average of 2.25 nonLegionella killed half the embryos. There is no significant difference between these LD_{50s}. Embryo deaths were due primarily to the nonLegionella. The role of *L. pneumophila* was of minor consequence.

Table 1. First Inoculation of the Water System

Date	PV Count *L.p.* cells/ml	Plate Count *L.p.* colonies/ml
2-9-96 (20 min. after inoculation)	2023	260
2-15-96	1218	0
2-23-96	322	0
3-1-96 (a.m.)	308	0

The water system was inoculated on 2-9-96 with 2 x 10^4 *Legionella pneumophila* cells/ml (L.p. cells/ml). Cell counts were determined microscopically using polyvalent (PV) staining and by culture on BCYE agar plates.

Figure 3. Cell Counts Following First Inoculation



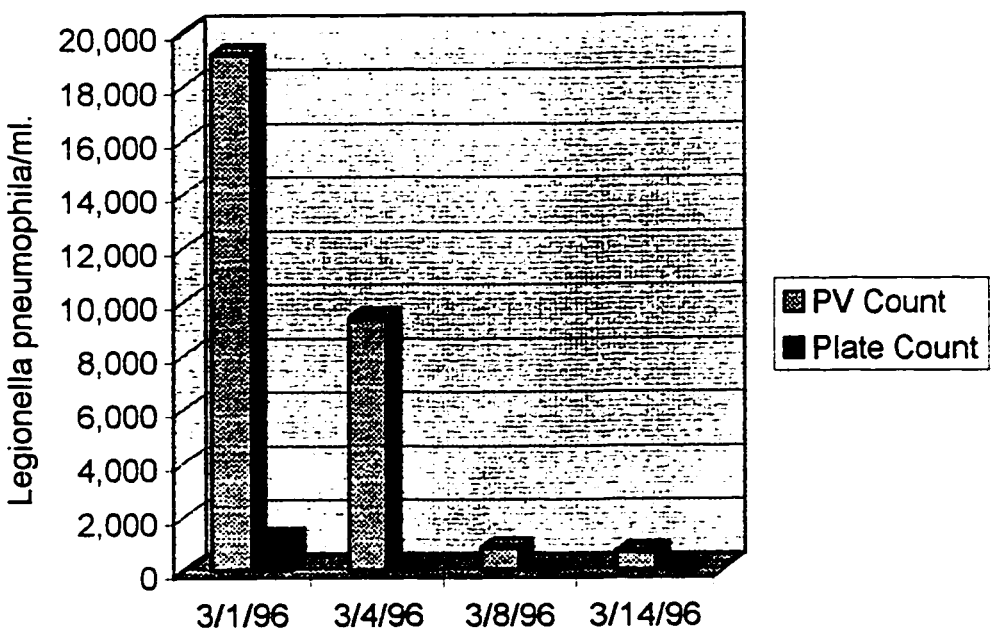
The *Legionella pneumophila* polyvalent (PV) count and plate count on BCYE agar were determined following the 2-9-96 inoculation of the water system.

Table 2. Second Inoculation of the Water System

Date	PV Count *L.p.* cells/ml	Plate Count *L.p.* colonies/ml
3-1-96 (20 min after inoculation)	19,180	1217
3-4-96	9380	0
3-8-96	840	0
3-14-96 (a.m.)	700	0

The water system was inoculated on 3-1-96 (p.m.) with 6 x 10^4 *Legionella pneumophila* cells/ml (*L.p.* cells/ml). Cell counts were determined microscopically using polyvalent (PV) staining and by culture on BCYE agar plates.

Figure 4. Cell Counts Following Second Inoculation



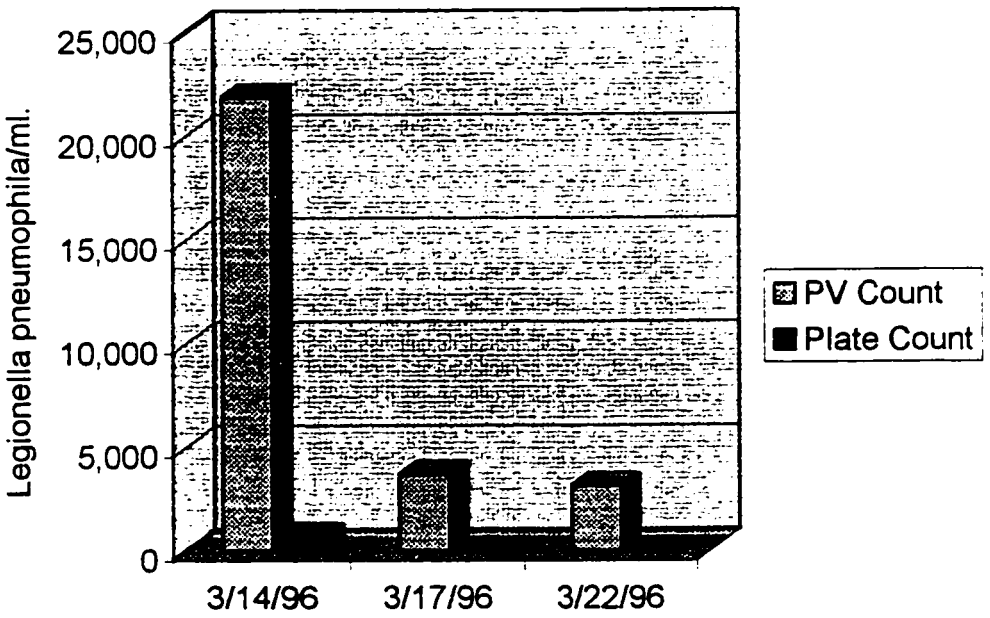
The *Legionella pneumophila* polyvalent (PV) count and plate count on BCYE agar were determined following the 3-1-96 inoculation of the water system.

Table 3. Third Inoculation of the Water System

Date	PV Count *L.p.* cells/ml	Plate Count *L.p.* colonies/ml
3-14-96 (20 min. after inoculation)	21.910	715
3-17-96	3722	0
3-22-96 (a.m.)	3150	0

The water system was inoculated on 3-14-96 (p.m.) with 6 x 10^4 *Legionella pneumophila* cells/ml (*L.p.* cells/ml). Cell counts were determined microscopically using polyvalent (PV) staining and by culture on BCYE agar plates.

Figure 5. Cell Counts Following Third Inoculation



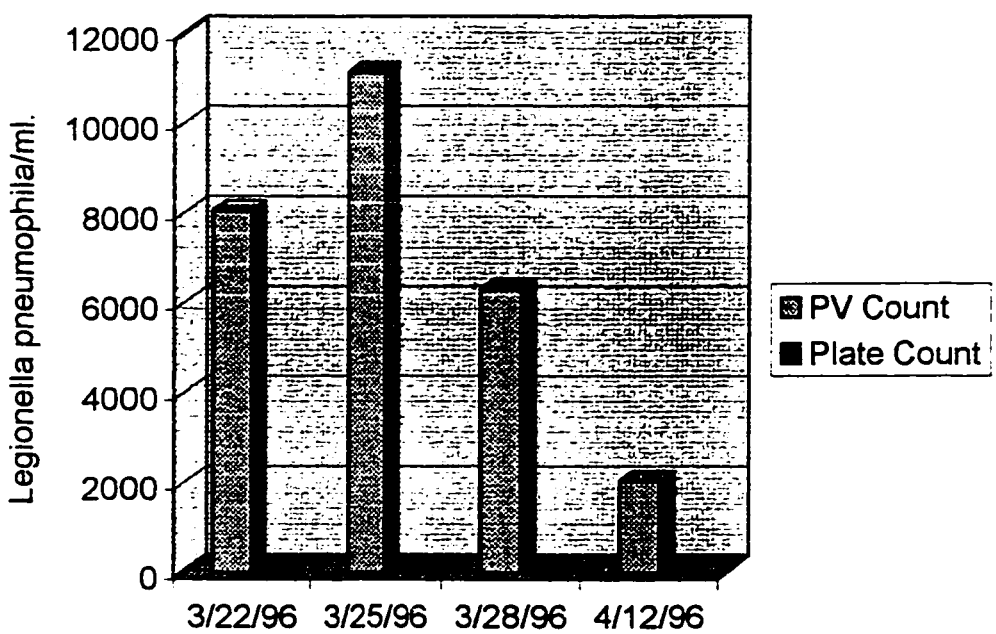
The *Legionella pneumophila* polyvalent (PV) count and plate count on BCYE agar were determined following the 3-14-96 inoculation of the water system.

Table 4. Fourth Inoculation of the Water System

Date	PV Count *L.p.* cells/ml	Plate Count *L.p.* colonies/ml
3-22-96 (20 min. after inoculation)	8050	0
3-25-96	11.130	1
3-28-96	6300	3
4-12-96	2030	0

The water system was inoculated on 3-22-96 (p.m.) with 6 x 10^4 *Legionella pneumophila* cells/ml (*L.p.* cells/ml). Cell counts were determined microscopically using polyvalent (PV) staining and by culture on BCYE agar plates.

Figure 6. Cell Counts Following Fourth Inoculation



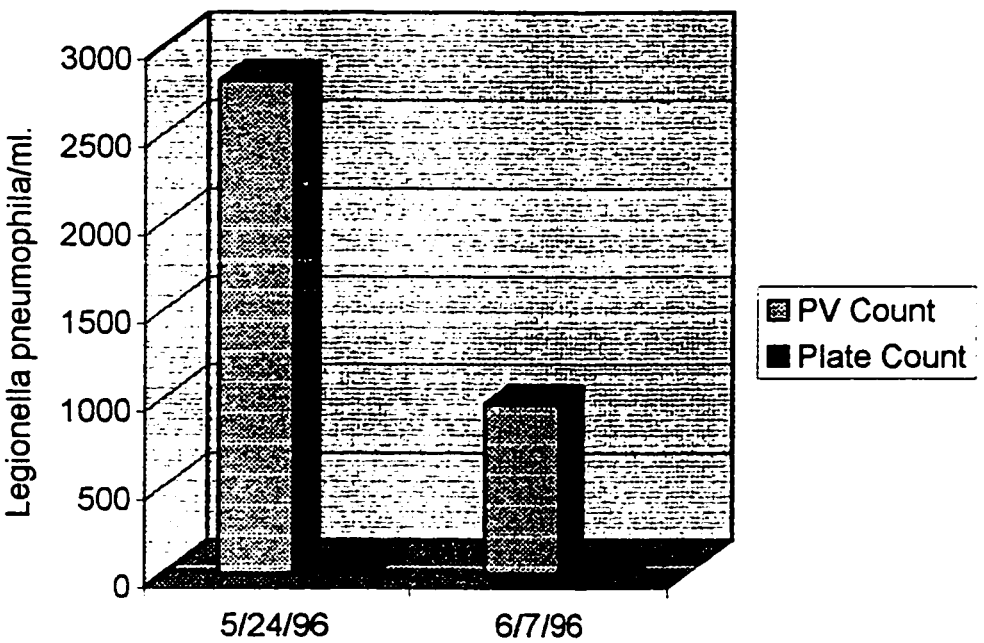
The *Legionella pneumophila* polyvalent (PV) count and plate count on BCYE agar were determined following the 3-22-96 inoculation of the water system.

Table 5. Fifth Inoculation of the Water System

Date	PV Count *L.p.* cells/ml	Plate Count *L.p.* colonies/ml
5-24-96	2800	0
6-7-96	966	0

The water system was inoculated on 5-3-96 with 6 x 10^4 *Legionella pneumophila* cells/ml (*L.p.* cells/ml). Cell counts were determined microscopically using polyvalent (PV) staining and by culture on BCYE agar plates.

Figure 7. Cell Counts Following Fifth Inoculation



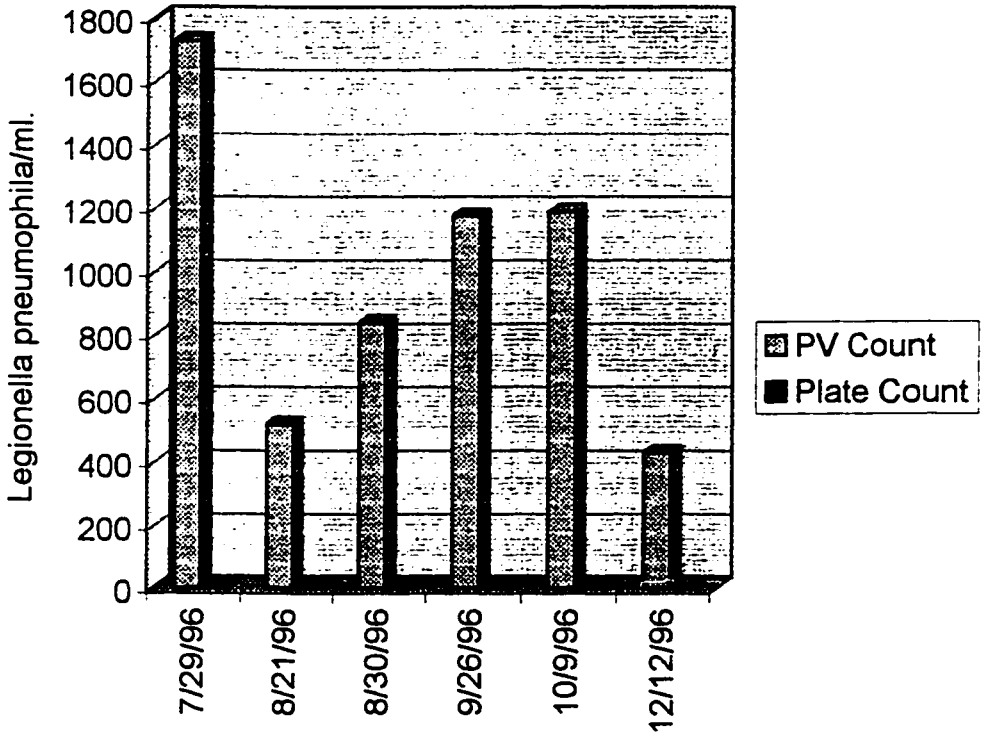
The *Legionella pneumophila* polyvalent (PV) count and plate count on BCYE agar were determined following the 5-3-96 inoculation of the water system.

Table 6. Sixth Inoculation of the Water System

Date	PV Count *L.p.* cells/ml	Plate Count *L.p.* colonies/ml
7-29-96	1730	0
8-21-96	518	0
8-30-96	840	0
9-26-96	1177	0
10-9-96	1190	0
12-12-96	430	0

The water system was inoculated on 7-23-96 with 1.2×10^5 *Legionella pneumophila* cells/ml (*L.p.* cells/ml). Cell counts were determined microscopically using polyvalent (PV) staining and by culture on BCYE agar plates.

Figure 8. Cell Counts Following Sixth Inoculation



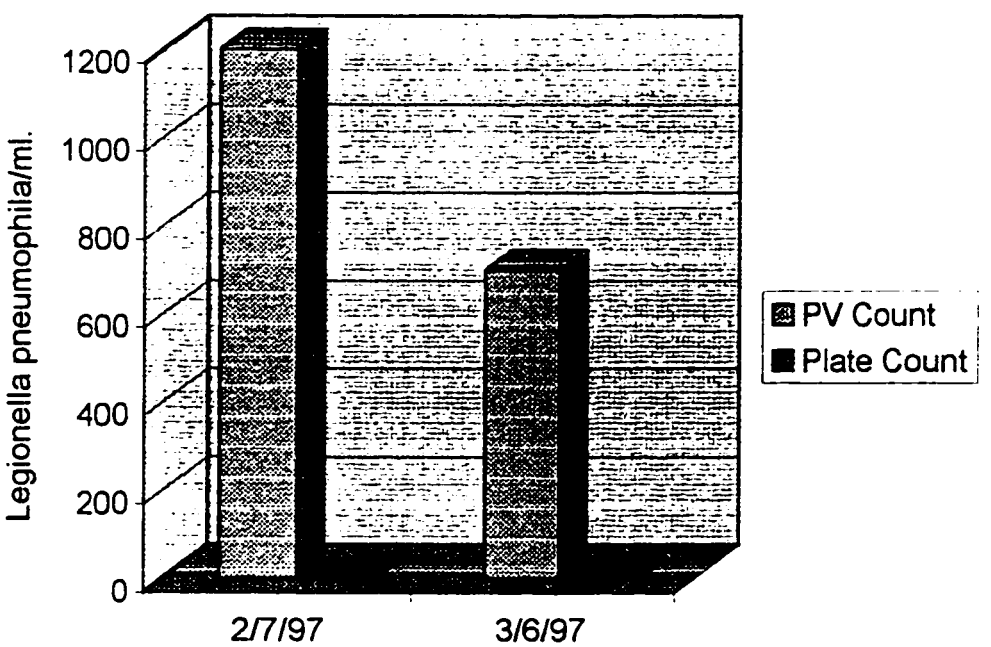
The *Legionella pneumophila* polyvalent (PV) count and plate count on BCYE agar were determined following the 7-23-96 inoculation of the water system.

Table 7. Seventh Inoculation of the Water System

Date	PV Count *L.p.* cells/ml	Plate Count *L.p.* colonies/ml
2-7-97	1200	0
3-6-97	700	0

The water system was inoculated on 12-24-96 with 1.2×10^5 *Legionella pneumophila* cells/ml (*L.p.* cells/ml). Cell counts were determined microscopically using polyvalent (PV) staining and by culture on BCYE agar plates.

Figure 9. Cell Counts Following Seventh Inoculation



The *Legionella pneumophila* polyvalent (PV) count and plate count on BCYE agar were determined following the 12-24-96 inoculation of the water system.

Table 8. Effect of nonLegionella Bacteria on *Legionella pneumophila* Culturability

Bottle #1 [sterile filtered hospital water and an estimated 100 *Legionella pneumophila* (L.p.)/ml]

Date	BCYE Agar (*L.p.*/ml)	Plate Count Agar (nonL.p./ml)	Polyvalent Count (*L.p.*/ml)
10-3-97	40	0	N.D.
10-11-96	16	0	N.D.
10-18-96	6	0	N.D.
10-25-96	4	0	N.D.
11-1-96	2	0	82

N.D. not determined

Bottle #2 [sterile filtered hospital water, an estimated 100 *L.p.*/ml and an estimated 100 nonL.p./ml]

Date	BCYE Agar (*L.p.*/ml)	Plate Count Agar (nonL.p./ml)	Polyvalent Count (*L.p.*/ml)
10-3-96	50	15	N.D.
10-11-96	10	too numerous to count	N.D.
10-18-96	0	too numerous to count	N.D.
10-25-96	2	too numerous to count	N.D.
11-1-96	0	too numerous to count	103

N.D. not determined

Table 9. INT-Formazan Formation in Hospital *Legionella pneumophila* Cells

INT Incubation Time (hrs)	# INT-F Positive Cells	# INT-F Negative Cells	% Cells with INT-F Formation
1	23	2	92
2.5	22	3	88

The *Legionella pneumophila* cells were one time freshly cultured. At 2.5 hours formazan deposits were larger and easier to detect.

Figure 10. INT-Formazan Formation in Hospital *Legionella pneumophila* Cells

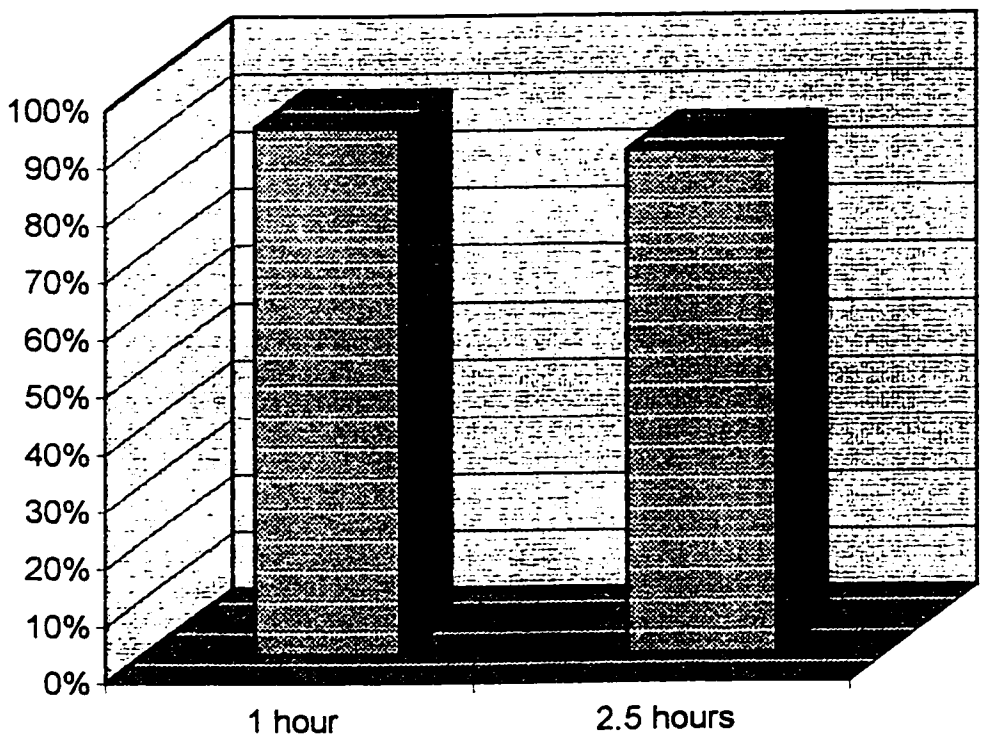


Table 10. INT-Formazan Formation in Water System Cells

Water System *Legionella pneumophila* Planktonic Cells

Date	INT Incubation Time (hrs)	# INT-F Positive Cells	# INT-F Negative Cells	% Cells INT-F Formation
9-26-96	3	5	5	50
10-9-96	5	4	6	40
10-11-96	5	10	15	40
10-25-96	5	5	5	50

Water System *Legionella pneumophila* Biofilm Cells

Date	INT Incubation Time (hrs)	# INT-F Positive Cells	# INT-F Negative Cells	% Cells INT-F Formation
10-11-96	5	24	6	80
10-25-96	5	15	15	50

Figure 11. INT-Formazan Formation in Water System Cells

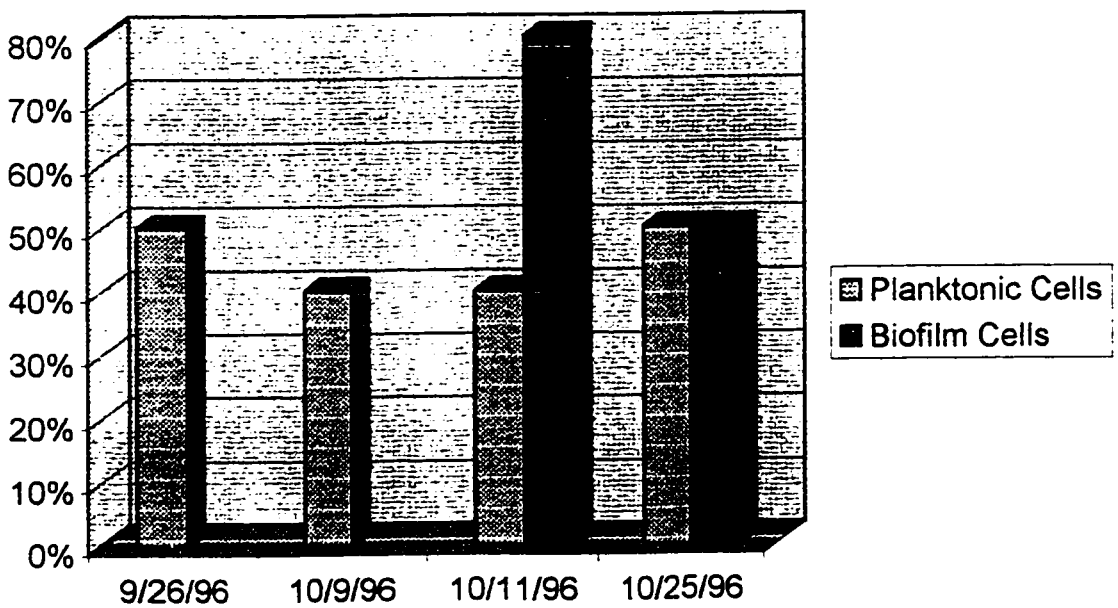


Table 11. INT-Formazan Formation – Bottle Experiment

Pure *Legionella pneumophila* was put into sterile bottles.

Bottle 1

Cell Type	INT Incubation Time (hrs)	# INT-F Positive Cells	# INT-F Negative Cells	% Cells INT-F Formation
Planktonic	5	0	10	0
Biofilm	5	3	7	30

Bottle 2

Cell Type	INT Incubation Time (hrs)	# INT-F Positive Cells	# INT-F Negative Cells	% Cells INT-F Formation
Planktonic	5	0	10	0
Biofilm	5	5	5	50

Figure 12. INT-Formazan Formation – Bottle Experiment

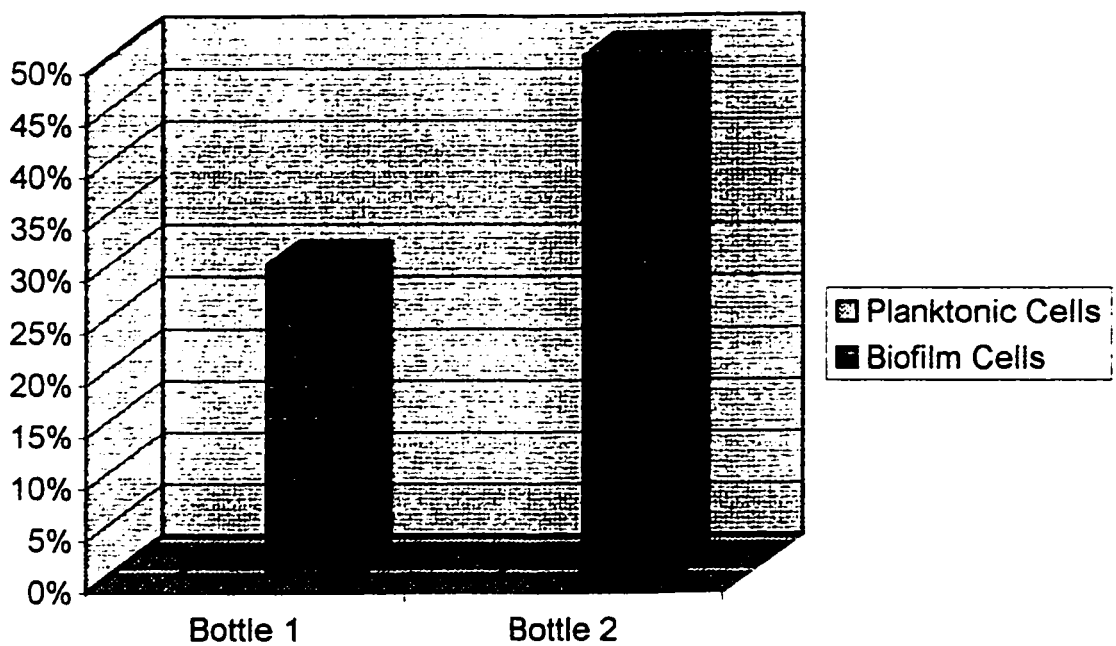


Table 12. Effect of One Time Cultured Hospital *Legionella pneumophila* (*L.p.*) Cells on Embryonated Eggs

Date of Inoculation 6-9-95

# Eggs Inoculated	Inoculum *L.p.*/ml	# Embryos Dead Day 10 or Later	# Embryos Alive	#Embryos Nonspecific Death
4	none (control)	0	3	1
8	1×10^3	5	0	3
8	1×10^2	3	1	4
12	10	0	5	7
12	1	1	4	7

The presence of *L.p.* in harvested yolk sacs was confirmed by culture and polyvalent staining.

Date of Inoculation 2-7-97

#Eggs Inoculated	Inoculum *L.p.*/ml	# Embryos Dead Day 10 or Later	# Embryos Alive	# Embryos Nonspecific Death
7	2×10^2	5	1	1
8	20	3	4	1
8	2	1	3	4
7	0.2	0	5	2

The presence of *L.p.* in harvested yolk sacs was confirmed by culture and polyvalent staining.

Date of Inoculation 4-3-97

#Eggs Inoculated	Inoculum *L.p.*/ml	# Embryos Dead Day 10 or Later	# Embryos Alive	# Embryos Nonspecific Death
8	3.4×10^2	8	0	0
8	34	4	4	0
8	3.4	1	7	0
7	0.34	0	6	1

The presence of *L.p.* in harvested yolk sacs was confirmed by culture and polyvalent staining.

Table 13. Infectivity of Hospital *Legionella pneumophila* Cells

Date	# Eggs Inoculated Minus Nonspecific Deaths	Inoculum *L.p.*/ml	# Embryos Dead Day 10 or Later	Mortality Rate	LD_{50} *L.p.*/egg
6-9-95	8 – 4 = 4	1×10^2	3	75%	35.2
	12 – 7 = 5	10	0	0%	
2-7-97	7 – 1 = 6	2×10^2	5	83.3%	26.2
	8 – 1 = 7	20	3	42.9%	
4-3-97	8 – 0 = 8	34	4	50%	17

The lethal dose $_{50}$ (LD_{50}) was determined in embryonated eggs inoculated with one time cultured hospital *Legionella pneumophila* (*L.p.*) cells using the proportional distance formula in the Reed and Muench method.

Table 14. Effect of One Time Cultured nonLegionella (nonL.p.) Bacteria Planktonic Cells on Embryonated Eggs

Date of Inoculation 12-12-96

# Eggs Inoculated	Inoculum nonL.p./ml	# Embryos Dead Day 10 or Later	#Embryos Alive	#Embryos Nonspecific Death
5	none (control)	0	3	2
10	8×10^2	9	0	1
10	80	5	3	2
10	8	6	0	4
10	0.8	1	7	2

The presence of nonL.p. in harvested yolk sacs was confirmed by culture and Gram staining.

Date of Inoculation 2-7-97

# Eggs Inoculated	Inoculum nonL.p./ml	# Embryos Dead Day 10 or Later	# Embryos Alive	# Embryos Nonspecific Death
7	9.2×10^2	5	0	2
8	92	6	0	2
8	9.2	6	1	1
7	0.92	0	6	1

The presence of nonL.p. in harvested yolk sacs was confirmed by culture and Gram staining.

Date of inoculation 3-6-97

# Eggs Inoculated	Inoculum nonL.p./ml	# Embryos Dead Day 10 or Later	# Embryos Alive	# Embryos Nonspecific Death
7	8.4×10^1	3	0	4
7	8.4	1	0	6
7	0.84	0	5	2

The presence of nonL.p. in harvested yolk sacs was confirmed by culture and Gram staining.

Table 15. Infectivity of nonLegionella Bacteria

Date	# Eggs Inoculated Minus Nonspecific Deaths	Inoculum nonL.p./ml	#Embryos Dead Day 10 or Later	Mortality Rate	LD_{50} nonL.p./egg
12-12-96	10 – 4 = 6	8	6	100%	1.95
	10 – 2 = 8	0.8	1	12.5%	
2-7-97	8 – 1 = 7	9.2	6	85.7%	2.86
	7 – 1 = 6	0.92	0	0%	
3-6-97	7 – 6 = 1	8.4	1	100%	2.31
	7 – 2 = 5	0.84	0	0 %	

The lethal dose $_{50}$ (LD_{50}) was determined in embryonated eggs inoculated with one time cultured nonLegionella (nonL.p.) bacteria (planktonic cells) using the proportional distance formula in the Reed and Muench method.

Table 16. Effect of Water System Planktonic Cells [Mixture of *Legionella pneumophila* (*L.p.*) and nonLegionella Bacteria] on Embryonated Eggs

Date of Inoculation 5-24-96

# Eggs Inoculated	Inoculum *L.p.*/ml	# Embryos Dead Day 10 or Later	# Embryos Alive	# Embryos Nonspecific Death
5	none (control)	1	4	0
8	2.8×10^2	7	0	1
8	28	6	0	2
8	2.8	3	3	2
7	0.28	0	6	1

Legionella pneumophila were not recovered from harvested yolk sacs.

Date of Inoculation 2-7-97

# Eggs Inoculated	Inoculum *L.p.*/ml	# Embryos Dead Day 10 or Later	# Embryos Alive	# Embryos Nonspecific Death
3	none (control)	0	3	0
7	1.2×10^2	6	0	0
8	12	7	0	1
8	1.2	5	2	1
7	0.12	1	5	1

Legionella pneumophila was not recovered from harvested yolk sacs.

Date of Inoculation 3-6-97

# Eggs Inoculated	Inoculum *L.p.*/ml	# Embryos Dead Day 10 Or Later	# Embryos Alive	# Embryos Nonspecific Death
7	70	7	0	0
7	7	5	0	2
7	0.7	2	3	2

Legionella pneumophila was not recovered from harvested yolk sacs.

Table 17. Infectivity of Water System Planktonic Cells

Date	# Eggs Inoculated Minus Nonspecific Deaths	Inoculum *L.p.*/ml	# Embryos Dead Day 10 or Later	Mortality Rate	LD_{50} *L.p.*/egg
5-24-96	8 – 2 = 6	2.8	3	50%	1.4
2-7-97	8 – 1 = 7	1.2	5	71.4%	0.39
	7 – 1 = 6	0.12	1	16.7%	
3-6-97	7 – 2 = 5	7	5	100%	0.88
	7 – 2 = 5	0.7	2	40%	

The lethal dose $_{50}$ (LD_{50}) was determined in embryonated eggs inoculated with water system planktonic cells, a mixture of *Legionella pneumophila* (*L.p.*) and nonLegionella bacteria using the proportional distance formula in the Reed and Muench method.

Table 18. Effect of Water System Biofilm Cells [Mixture of *Legionella pneumophila (L.p.)* and nonLegionella Bacteria] on Embryonated Eggs

Date of Inoculation 3-6-97

Right Coupon

# Eggs Inoculated	Inoculum *L.p.*/ml	# Embryos Dead Day 10 or Later	# Embryos Alive	# Embryos Nonspecific Death
3	none (control)	0	3	0
7	81	6	1	0
7	8.1	2	4	1
7	0.81	0	7	0
7	0.081	0	6	1

Legionella pneumophila was not recovered from harvested yolk sacs.

Date of inoculation 3-6-97

Left Coupon

# Eggs Inoculated	Inoculum *L.p.*/ml	# Embryos Dead Day 10 or Later	# Embryos Alive	# Embryos Nonspecific Death
7	52	0	4	3
7	5.2	3	3	1
7	0.52	0	5	2

Legionella pneumophila was not recovered from harvested yolk sacs.

Date of Inoculation 4-3-97

# Eggs Inoculated	Inoculum *L.p.*/ml	# Embryos Dead Day 10 or Later	# Embryos Alive	# Embryos Nonspecific Death
5	none (control)	0	5	0
10	45	6	1	3
10	4.5	1	7	2
10	0.45	0	8	2

Legionella pneumophila was not recovered from harvested yolk sacs.

Table 19. Infectivity of Water System Biofilm Cells

Date	# Eggs Inoculated Minus Nonspecific Deaths	Inoculum *L.p.*/ml	# Embryos Dead Day 10 or Later	Mortality Rate	LD_{50} *L.p.*/egg
(Right Coupon)					
3-6-97	7 – 0 = 7	81	6	85.7%	15.7
	7 – 1 = 6	8.1	2	33.3%	
(Left Coupon)					
3-6-97	7 – 1 = 6	5.2	3	50%	2.6
4-3-97	10 – 3 = 7	45	6	85.7%	12.6
	10 – 2 = 8	4.5	1	12.5%	

The lethal dose $_{50}$ (LD_{50}) was determined in embryonated eggs inoculated with water system biofilm cells, a mixture of *Legionella pneumophila* (*L.p.*) and nonLegionella bacteria using the proportional distance formula in the Reed and Muench method.

Table 20. Summary of Lethal Dose$_{50s}$ (# cells/egg)

Hospital Cells Pure one time cultured L.p. Added to water system	nonL.p.(Planktonic Cells) One time cultured From water system	Water System (Planktonic Cells)		Water System (Biofilm Cells)	
		L.p.cells/egg	nonL.p.cells/egg	L.p.cells/egg	nonL.p.cells/egg
35.2	1.95	1.4	2.9	15.7	0.7
26.2	2.86	0.39	0.55	2.6	11.3
17	2.31	0.88	3.3	12.6	6.1
Mean 26.1	Mean 2.37	Mean 0.89	Mean 2.25	Mean 10.3	Mean 6.03

DISCUSSION

The results of this research can be summarized as follows:

1. A water system was constructed to study the viable nonculturable phenomenon in environmental populations of *L. pneumophila*.

   - During the twenty-four months of continuous operation, it simulated the natural environment found in health care facilities and served as a source of planktonic and biofilm cells.

2. It has been observed by many researchers that bacteria can enter into a viable nonculturable state.

   - Both planktonic and biofilm *L. pneumophila* in the water system existed primarily as nonculturable cells.

   - A portion of nonculturable planktonic and biofilm *L. pneumophila* cells were shown to be viable using the INT assay.

   - The long term persistence of *L. pneumophila* in the water system indicates its viability in a nonculturable state.

3. Several investigations have shown that *L. pneumophila* requires nutrients supplied by other microorganisms.

   - NonLegionella bacteria which coexisted with *L. pneumophila* in the water system had no effect on its culturability.

4. The virulence of L. pneumophila is often determined by inoculation of cells into embryonated chicken eggs.

   - A mixture of planktonic cells (*L. pneumophila* and nonLegionella) from the water system were more virulent than a mixture of biofilm cells.

   - Embryo deaths were due primarily to the nonLegionella, and *L. pneumophila* had little or no impact.

   - Published reports of studies where embryonated eggs are inoculated with environmental samples and the virulence of the mixture attributed to the *L. pneumophila* in the sample should be reevaluated.

In laboratories microbes are generally grown in artificial high nutrient environments. These conditions are not representative of the natural environment of water organisms found in health care facilities. The water system constructed for this study mimicked the conditions that exist in a domestic hot water system. The water system was a substantial improvement over previous systems and served as a source of planktonic and biofilm *Legionella pneumophila* cells. The cPVC tubing used in this system was not inhibitory to *L. pneumophila* survival as is new copper piping. The EPDM coupons were readily colonized by microbes as are many rubber components in a domestic water system. Temperature was maintained between 110-120° F (43-49° C) which approximates the hot water temperature in health care facilities. The mixed population of bacteria in this study was a realistic representation of the way *L. pneumophila* is found in nature in comparison to studies where pure cultures of *L. pneumophila* have been used.

The ability to remain viable during a period of starvation is essential to the survival of many bacterial species. *E. coli* undergoes a significant molecular realignment in response to starvation which increases its resistance to this stress (Groat et al., 1986). At the onset of starvation, *E. coli* synthesizes about thirty proteins which have been called starvation proteins. Many of these proteins are unique to the starvation condition and are not synthesized during growth (Groat and Matin, 1986). Some of these proteins may be involved in degradation of growth associated proteins and others may be sigma factors involved in control of transcription of starvation response genes as observed in *Bacillus subtilis* (Trempy

et al., 1985; Reeve et al., 1984). During starvation the organisms must still retain the ability to transport any substrate into the cell which can be utilized as an energy source (Koch, 1971). As part of their survival strategy, bacteria may lose the ability to grow on culture media but still retain viability.

When *L. pneumophila* was added to the water system it became nonculturable within three days. Biofilm cells scraped off of the EPDM coupons were also nonculturable. Selective media, DGVP, and direct placement of a polycarbonate filter, through which a 25 ml water system sample was filtered, onto the surface of the medium did not recover any culturable *L. pneumophila* cells.

To show that the nonculturable cells in the water system were not dead but still viable, the INT assay was used. The assay indicates individual cell viability through the demonstration of an active electron transport system. INT removes hydrogen atoms from an active electron transport system, and the INT is reduced to red deposits of INT-formazan within the cell.

Viability was demonstrated in a portion of the nonculturable planktonic and biofilm *L. pneumophila* cells from the water system. Culturable *L. pneumophila* cells used for inoculation of the system demonstrated the greatest respiratory rate. When these cells were stressed in the water system and became nonculturable, electron transport activity was reduced in both planktonic and biofilm cells. No significant difference between planktonic and biofilm cells was discernable.

In this study the INT assay was improved for use with *Legionella* samples. Microbes under starvation conditions could not be expected to have a rapid rate of

respiration; consequently, INT-formazan deposits in the cells are small and difficult to detect. To stimulate electron transport activity, the assay was modified to incorporate resuscitation.

Resuscitation procedures have been attempted with nonLegionella bacteria. Roszak et al., (1984) resuscitated viable but nonculturable *Salmonella enteritidis* to a culturable state by the addition of nutrients to their samples. *Vibrio vulnificus* was resuscitated to the culturable state not by the addition of nutrients but rather by an increase in incubation temperature (Nilsson et al., 1991). Colwell et al., (1985) resuscitated *Vibrio cholerae* and *E. coli* by inoculation into ligated intestinal loops of rabbits.

In this research resuscitation of nonculturable *L. pneumophila* back to culturability was not achieved. However, resuscitation of the organism to a more metabolically active state occurred when yeast extract broth was incorporated into the INT assay. Use of the broth increased the size of INT-F deposits making detection of viable cells easier. The increased size of the formazan deposits indicates that electron transport was occurring at a more rapid rate. Trevors et al., (1982) increased ETS activity in soil samples with either yeast extract broth or glucose. Olanczuk-Neyman and Vosjan (1977) increased ETS activity in marine sediments by using NADH, NADPH and succinate in the assay. In this study stimulation of respiration in *L. pneumophila* using NADH, NADPH and succinic acid was unsuccessful.

In the natural environment organisms are subjected to variations in nutrient

availability. Despite low nutrient conditions microbes do survive. For a cell to survive during starvation, only a very small part of its metabolic potential needs to be expressed. These maintenance functions include maintenance of osmotic potential, accumulation of a higher concentration of substrates in the cell than exists in the medium, turnover of cell materials and maintenance of a membrane potential. None of these functions results in increased mass. If ATP is available to the cell at a low rate, the nongrowth associated demand for ATP or maintenance energy must be met before energy can be expended for growth (Mason et al., 1986; Ingraham et al., 1983). Maintenance energy is required under both growth and starvation conditions (Dawes, 1976).

Although the water system was inoculated on seven occasions, there was an interval of five months where no *L. pneumophila* were added to the system. The *Legionella* cells were detectable during this period by polyvalent staining but not by culture. The long term persistence of the organism indicates available energy diverted to maintenance functions, but not enough energy was present for growth in the system. The *L. pneumophila* was present in a viable but nonculturable state. Available energy can come from compounds released when other members of the population undergo lysis, a process known as cryptic growth (Mason, 1986).

L. pneumophila has been shown to be incapable of growth in sterile water without the presence of other microorganisms. These organisms produce nutrients required for *L. pneumophila* growth. In this project one time cultured *L. pneumophila* inoculated into sterile filtered hospital water lost culturability at the

same rate as when nonLegionella bacteria from the water system were also included in the microcosm. In this experiment nonLegionella bacteria had no effect on the viable but nonculturable phenomenon.

The virulence of *L. pneumophila* is often determined by inoculation of cells into embryonated chicken eggs. In this research several controls were used in virulence testing. From every batch of eggs used for inoculation, several eggs were always uninoculated and served as untouched controls to determine if embryos used in the experiment were healthy and capable of further development. Into some eggs a syringe needle was inserted with no inoculum being injected and into other eggs sterile deionized water was injected to determine if either the inoculation procedure or the deionized water used to make bacterial dilutions affected the embryos. Autoclaved planktonic cells from the water system and also autoclaved biofilm cells were inoculated into eggs as controls. Neither deionized water nor autoclaved cells had any appreciable effect on the embryos.

The lethal dose $_{50}$ can be used as a measure of virulence. Ten fold bacterial dilutions were inoculated into embryonated eggs to determine the dilution at which 50% of the embryos died. If the 50% point fell between two dilutions, the LD_{50} was calculated using the proportional distance formula in the Reed and Muench method. It is recognized that probit analysis would provide a more extensive indicator of LD_{50} values, but it would require multiple samples of lethality at a variety of dose levels across the entire spectrum of dosages (Salsburg, 1986). Within the limits of available resources, the still widely utilized Reed and

Muench method was used to determine the 50% end points.

LD_{50} values indicated that the one time cultured hospital *L. pneumophila* cells which were added to the water system were not as virulent as the cultured nonLegionella bacteria which developed in the system, LD_{50} mean values of 26.1 cells per egg versus 2.37 cells per egg respectively. Analysis of LD_{50} values also revealed that a mixture of planktonic cells (*L. pneumophila* and nonLegionella) from the water system were more virulent than a mixture of biofilm cells; fewer planktonic cells than biofilm cells were needed to kill 50% of the embryos.

A comparison was made between the LD_{50s} of planktonic nonLegionella cells alone and a mixture of planktonic *L. pneumophila* and nonLegionella cells. It took an average of 2.37 pure nonLegionella cells per egg to exert the desired death rate. In inocula consisting of a mixture of *L. pneumophila* and nonLegionella, an average of 2.25 nonLegionella cells exerted the same kill. This is not a significant difference in LD_{50} values indicating that embryo deaths were due primarily to the nonLegionella and that *L. pneumophila* had little or no impact.

The ability of *L. pneumophila* to grow well in embryonated eggs is documented (McDade et al., 1977). However, embryos are also susceptible to other bacteria, and deaths occurring during the first three days following inoculation are considered nonspecific deaths from causes other than infection with *L. pneumophila*. The presence of *L. pneumophila* in yolk sacs harvested from the embryos that died after the third day following inoculation must be confirmed.

In some studies death of the embryo is ascribed to *L. pneumophila* when microscopic examination of stained yolk sac smears reveals a minimum of just one of these organisms in 100 microscopic fields (McDade and Shepard, 1979). Studies where embryonated eggs are inoculated with environmental samples and the virulence of the mixture attributed to the *L. pneumophila* in the sample should be reevaluated.

This study demonstrated viable but nonculturable *L. pneumophila* persisting in a water system which mimicked the hot water systems found in health care facilities. These organisms were found in the bulk water and also attached to surfaces as part of biofilms. Although no longer recoverable by ordinary microbiological techniques, inhalation of an aerosol contaminated with these cells could result in infection of a susceptible host.

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ABSTRACT

A STUDY OF THE RELATIONSHIP BETWEEN VIABILITY, CULTURABILITY AND VIRULENCE IN ENVIRONMENTAL POPULATIONS OF *LEGIONELLA PNEUMOPHILA*

by

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Many researchers have observed that bacteria can enter into a viable but nonculturable state. In order to study this phenomenon in *Legionella pneumophila*, a model hot water system was constructed to serve as a source of planktonic and biofilm cells. Both planktonic and biofilm *L. pneumophila* existed primarily as nonculturable cells. A portion of these nonculturable cells were shown to be viable using the tetrazolium salt, INT, which is an indicator of respiratory activity. The nonLegionella bacteria which coexisted with *L. pneumophila* in the water system had no effect on its culturability. Inoculation of embryonated chicken eggs indicated that a mixture of planktonic cells (*L. pneumophila* and nonLegionella) from the water system were more virulent than a mixture of biofilm cells. However, embryo deaths were due primarily to the nonLegionella, and *L. pneumophila* had little or no impact. Published reports of

studies where embryonated eggs are inoculated with environmental samples and the virulence of the mixture attributed to the *L. pneumophila* in the sample should be reevaluated.

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Publications
Chlorine, heat: popular ways to beat *Legionella.* J.W. Wireman, A. Schmidt, and D.T. Hutchins. Health Facilities Management. 1992. 5:28-34.

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